ENDOCRINOLOGY

VOLUME 60 APRIL, 1957 NUMBER 4

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IN VITRO STEROIDOGENESIS IN THE HUMAN NEONATAL ADRENAL GLAND, INCLUDING OBSERVATIONS ON HUMAN ADULT AND MONKEY ADRENAL GLANDS[1]

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THE unusual developmental pattern and large size of the human fetal adrenal gland has caused much speculation as to its function (1). The gland is proportionately by weight 10 to 20 times larger than the adult gland, and its large size is attributable to a histologically distinct portion, the fetal adrenal zone, which makes up about 80% of the gland at birth. During the first few months of postnatal life, this zone abruptly involutes, with vascular dilatation, hemorrhage, necrosis, removal of cellular debris, and collapse of the supporting fibrous stroma. The fetal zone is histochemically similar in some ways to the adult cortex and is presumably steroidogenic, although its function is unknown. The present study was designed to demonstrate whether or not the fetal gland as a whole was capable of *in vitro* steroidogenesis. Incubation techniques were employed to compare the human neonatal adrenal with adult human and monkey adrenals.

Steroids were readily demonstrated by a variety of tests in extracts of incubation media from both human adult and monkey glands, and 17-hydroxycorticosterone (Kendall's compound F) was found in extracts derived from each type of gland. By contrast, the incubation media from human newborn adrenals did not regularly contain measurable steroids. The infant glands were capable, however, of converting progesterone to

Received June 26, 1956.

[1] This investigation was supported by a research grant from the Association for Aid to Crippled Children and by research grant A-609 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

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17-hydroxycorticosterone, corticosterone (compound B), and possibly 11-desoxy, 17-hydroxycorticosterone (compound S). These studies indicate that the newborn adrenal has the ability to carry out certain hydroxylations on the steroid nucleus which are characteristic of the adult gland during steroidogenesis.

METHODS

Adrenal glands were obtained from etherized, exsanguinated, young adult monkeys, (Macacus cynomolgus), from male adult humans undergoing adrenalectomy for prostatic carcinoma, and from premature infants dying in the first few days of life. The glands were iced and sliced with a Stadie slicer. Attempts to dissect the fetal zone of the human infant glands free of adult-type cortex failed. The adrenal slices were incubated, usually for 4 to 6 hours, in Krebs-Ringer phosphate buffer with 200 mg. % glucose at an initial pH of 7.2 under an atmosphere of air, oxygen, or nitrogen. In one instance four human infant adrenals were minced and individually incubated for 6 to 17 days under sterile conditions in a medium made up of five parts Hanks' solution, four parts horse serum and one part chick embryo extract. ACTH, chorionic gonadotrophin, or vitamin C were added in certain experiments. Oxygen uptake was measured by the usual Warburg techniques, and Q_{O_2} was calculated (ml O_2 uptake/mg. tissue dry weight/hr.) for the second hour of incubation. After incubation for 3 to 6 hours, the media were removed and extracted with equal volumes of redistilled $CHCl_3$, once at pH7 and twice at pH1. The combined chloroform extracts were then washed once with cold 0.1N NaOH or 5% Na_2CO_3, and three times with water. The chloroform was removed by vacuum distillation at temperatures below 50° C and the dried residue taken up in absolute alcohol, diluted with water to 70% alcohol and partitioned three times against hexane. The combined alcohol fractions were extracted three times with $CHCl_3$ and dried as before. Tissue-free controls were invariably carried through the entire procedure and their assay values subtracted from the tissue extract assays. In a few instances, kidney tissue was run as an additional control. The chloroform residues were assayed by one or more of the following techniques, using 11-dehydro, 17-hydroxycorticosterone (compound E) or 17-hydroxycorticosterone as standards: ultraviolet absorption curves (UV) in a Beckman model DU spectrophotometer using the formula

$$D_{240\ m\mu} - \frac{D_{225\ m\mu} + D_{255\ m\mu}}{2}$$

for quantitation (2), periodic acid oxidation (FS) (3), Porter-Silber assay (4) and later the Silber-Porter assay (PS) (5) for 17,21-dihydroxy-20-ketones, thiosemicarbazide absorption at 302 mμ for Δ4,3-ketones (TSC) (6), Zimmermann assay (7), ketogenic steriod assay using chromic acid oxidation (8), paper chromatography, using the toluene-propylene glycol system of Zaffaroni and coworkers (9), spot testing with iodine-potassium iodide (I-KI), red triphenyltetrazolium (TPTZ) and dinitrophenylhydrazine (DNPH). Eluted bands were assayed by UV, PS, and sulfuric acid chromogen spectra (SAC) (10). In all spectrophotometric assays, an optical density value of <0.050 after subtraction of controls was regarded as insignificant. In the tables, these insignificant results are shown as "less than" a certain value; this value represents the limit of sensitivity of the method. Since these procedures give evidence but not proof that steroid material was present, the extracts will be referred to as containing steroid-like material (SLM).

RESULTS

(1) *Monkey adrenals.* Each pair of cynomolgus monkey adrenals yielded 120 to 350 mg. wet weight of adrenal slices. Except as indicated, each ex-

TABLE 1. INCUBATION OF MONKEY ADRENAL GLANDS WITH ACTH

Exp.	ACTH, I.U./cc.	UV*	FS*	PS*	TSC	GAS	Q_{O_2}
M1	1.0	105	150	<30	+	air	4.1
M2	3.3	93		19	+	air	1.2
M3	3.3	89	200			air	2.3
M4	3.3	51	74		0	air	1.8
M5	3.3	80	<23		+	air	2.5
M6	0.3		117			O_2	
M7	0.25	113	56	<10	+	O_2	
M8	3.3	95	33			O_2	2.3
M9	3.3	102				O_2	3.3
M10	3.3	77				O_2	3.9
M11	0.3	92				O_2	
	0.3	100				air	
M12	3.3	67	32			O_2	2.4
	3.3	90	24			air	1.3
M13	3.3	103	109			O_2	3.6
	3.3	44	65			air	1.4
M14	1.5	<17	<20		+	air	
	1.5	0	0		+	N_2	

* μg. 11-dehydro, 17-hydroxycorticosterone ≏/100 mg. wet weight of adrenal slices.

periment was run with glands from one monkey, sometimes divided into two aliquots to compare different systems. Direct extraction of unincubated adrenals yielded little SLM. Extracts from monkey adrenal slices incubated with ACTH in 14 experiments (M1 through M14, Table 1) yielded UV material in all of 13 trials, FS material in 9 of 10 trials, and positive TSC tests in 5 of 6 trials. Q_{O_2}'s with one exception (Experiment M1) ranged from 1.2 to 2.5 in air and from 2.3 to 3.9 in O_2; yields of SLM were not significantly different with the different Q_{O_2}'s. Yields diminished rapidly with time and practically ceased after 7 hours (Fig. 1). In Experiment

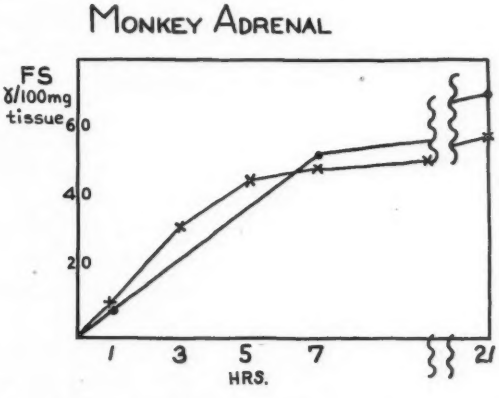

FIG. 1. Steroid-like material extracted from incubation of one pair of monkey adrenal glands. Yield diminished with time and practically ceased after 7 hours.

TABLE 2. MONKEY ADRENAL GLANDS INCUBATED WITH AND WITHOUT ACTH, ASCORBIC ACID, AND CHORIONIC GONADOTROPHIN

Exp.	ACTH, I.U./cc.	Ascorbic acid, mg./cc.	Chorionic gonadtrophin, I.U./cc.	UV*	FS*	Q_{O_2}
M3	3.3			89	200	2.3
	0			57	170	1.7
M4	3.3			51	74	1.8
	0			42	139	2.2
M5	3.3			80	<23	2.5
	0			26	<23	2.3
M6	0.3	1			38	
	0.3	0			117	
M8	3.3	1		109	<13	2.2
	3.3	0		95	33	2.3
M15			33	57	<18	2.3
			0	47	20	1.7

* μg. 11-dehydro, 17-hydroxycorticosterone = /100 mg. wet weight of adrenal slices.

M14, no UV or FS material was obtained after incubation under N_2, though an aliquot of the same tissue incubated under O_2 gave SLM by both tests; both aliquots, however, yielded a positive TSC test. Preliminary observations indicated that inclusion of ACTH probably increased the yields of SLM, but that ascorbic acid did not; a single trial with human chorionic gonadotrophin is included in the table (Table 2). Chromatographic analysis of combined aliquots from 8 experiments yielded a moderately intense band corresponding in position and SAC curve to 17-hydroxycorticosterone (Figs. 2, 3, Table 3); steroids indicated by other faint spot tests, one corresponding to corticosterone could not be further identified. With 200 μg. progesterone and 77 mg. of adrenal slices in the incubation medium in experiment M16, PS material was obtained indicating conversion to a dihydroxyketone in 27% yield (calculated as 17-hydroxycorticosterone). Tissue-free controls and adrenal tissue incubated without progesterone yielded no PS material. The extract from a similar experiment (M17) with 800 μg. progesterone and 225 mg. adrenal slices was chromatographed to yield three bands, two corresponding to 17-hydroxycorticosterone and corticosterone, and one unidentified. The intensity of the spot test indicated that most of the material corresponded to 17-hydroxycorticosterone. The material eluted from this band showed a UV absorption maximum at 240 mμ and a SAC spectrum corresponding to 17-hydroxycorticosterone (Table 3). No 240 mμ absorption maximum was obtained from the other two bands.

(2) *Human adults.* Five glands were obtained from 4 adults (Experiments HA1 through HA4). All these patients were receiving cortisone at the time of operation. In case HA1 (Table 4) UV and FS material was ob-

tained. In case HA2 (Table 4) UV material was obtained after incubation under air but not under N_2; no FS material was found. In case HA3, incubation of 1786 mg. slices with 1400 μg. of progesterone yielded PS material indicating conversion to a dihydroxyketone in 10% yield (calculated as 17-hydroxycorticosterone). Tissue-free controls and tissue incubated without progesterone yielded no PS material. In case HA4, both adrenals were obtained from the same individual at two operations 14 days apart. Extracts from both adrenals showed UV peaks at 240 mμ; these were combined and chromatographed. Two bands corresponding to 11-dehydro, 17-hydroxycorticosterone and 17-hydroxycorticosterone were located using TPTZ (Fig. 2); these bands were of approximately equal intensity. The band corresponding to 11-dehydro, 17-hydroxycorticosterone gave a blue color with I-KI. Material eluted from the 2 bands gave UV peaks at 240 mμ and SAC curves corresponding to 11-dehydro, 17-hydroxycorticosterone and 17-hydroxycorticosterone respectively (Fig. 4).

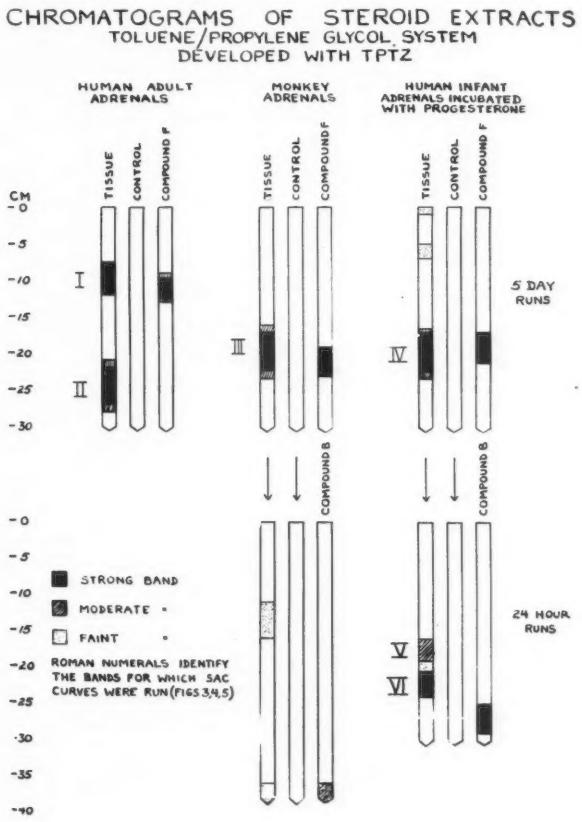

FIG. 2

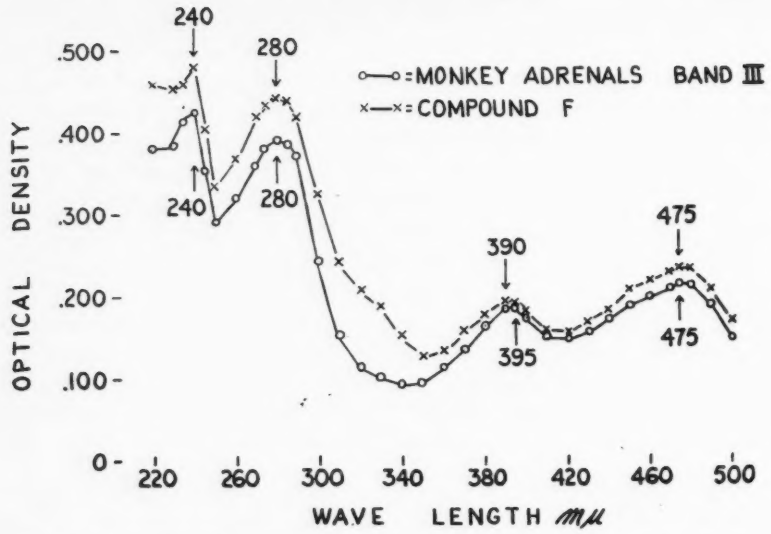

FIG. 3. Monkey adrenals; SAC spectra of Band III (Fig. 2) and standard 17-hydroxycorticosterone (Kendall's compound F).

(3) *Human infants.* Adrenal glands were obtained from 14 infants and fetuses weighing 350 to 2150 grams. Most infants had died within a few hours of birth, the oldest had lived for 8 days. The adrenals were removed at autopsy in from $\frac{3}{4}$ to $4\frac{1}{2}$ hours after death. After incubation of slices in 10 cases (Experiments HI1 through HI10, Table 5) only small amounts of UV and FS positive material were irregularly obtained from these glands. Q_{O_2}'s ranged from 1.0 to 2.4. In experiment HI11, pooled material from 4 human infant adrenals (total wet weight 3240 mg.) were incubated under

TABLE 3. EXPERIMENTS YIELDING STEROIDS IDENTIFIED BY SULFURIC ACID CHROMOGEN SPECTRA

Exp.	Gland	Weight of tissue incubated, mg.	Steroid added to incubation medium	Extracted steroids identified by SAC spectrum
Combined Aliquots from 8 Extracts	Monkey	Indeterminate	None	17-hydroxy-corticosterone
M17	Monkey	225	800 μg. progesterone	17-hydroxycorticosterone
HA4	Adult human	7204	None	11-dehydro, 17-hydroxy-corticosterone 17-hydroxycorticosterone
HI13	Infant human	2160	1000 μg. progesterone	17-hydroxycorticosterone corticosterone ? 11-desoxy, 17-hydroxy-corticosterone

TABLE 4. INCUBATION OF HUMAN ADULT ADRENAL GLANDS

Exp.	ACTH, I.U./cc.	Ascorbic acid, mg./cc.	UV*	FS*	Gas	Q_{O_2}
HA1	0	0	<7	<4	air	1.5
	3.3	0	10	<4	air	1.5
	3.3	0.3	28	14	air	1.5
HA2	3.3	0	20	<3	air	1.5
	3.3	0.3	24	<3	air	1.1
	3.3	0	<14	<3	N_2	0.1

* μg. 11-dehydro, 17-hydroxycorticosterone ≏/100 mg. wet weight of adrenal slices.

sterile conditions for 6 to 17 days in tissue culture medium and yielded 260 μg. of dehydroepiandrosterone equivalent (8 μg./100 mg. wet weight of adrenal tissue) in the Zimmermann assay. After chromic acid oxidation, the 17-ketogenic chromogens were 292 μg. androstanedione equivalents (these yields are corrected for tissue-free controls). Incubation of 476 mg. of adrenal slices from an 850 gm. infant (HI12) with 800 μg. of progesterone yielded PS material indicating conversion to a dihydroxyketone in 9% yield (calculated as 17-hydroxycorticosterone); neither tissue-free controls nor adrenal tissue incubated without progesterone yielded PS material. In a similar experiment (HI13) 1000 μg. of progesterone were incubated with 2160 mg. of adrenal slices from an 849 gm. infant and the extract chromatographed. Three bands corresponded in position with 17-hydroxycorticosterone, corticosterone, and 11-desoxy, 17-hydroxycorticosterone (Fig. 2). After elution, the amount of each measured by UV was 173 μg.,

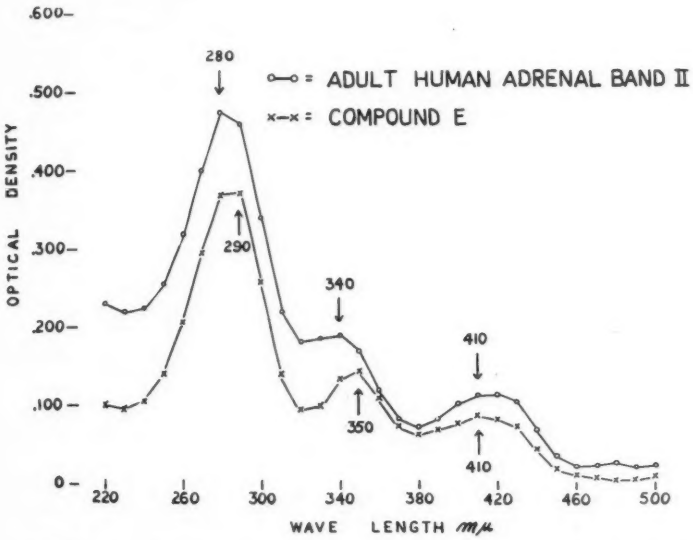

FIG. 4a. Human adult adrenals; SAC spectra of Band II (Fig. 2) and standard 11-dehydro, 17-hydroxycorticosterone (Kendall's compound E).

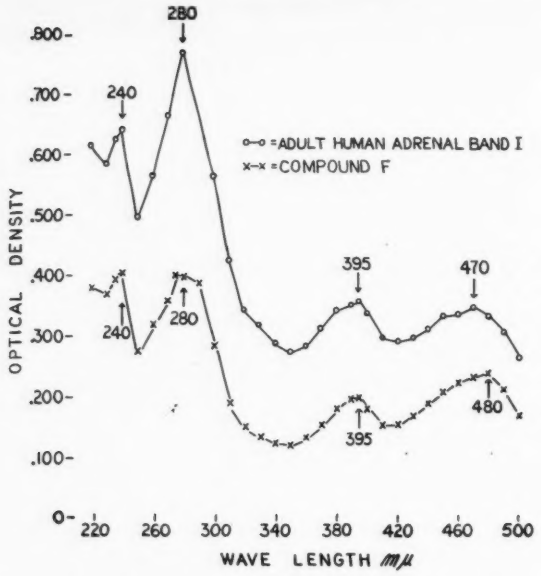

FIG. 4b. Human adult adrenals; SAC spectra of Band I (Fig. 2) and standard 17-hydroxycorticosterone (Kendall's compound F).

110 μg., and 25 μg. respectively, measured as 17-hydroxycorticosterone ⇔. SAC spectra corresponding to 17-hydroxycorticosterone and corticosterone respectively were obtained from the first two bands; the curve from the band corresponding to 11-desoxy, 17-hydroxycorticosterone was poorly defined. An aliquot of material identified as corticosterone was oxidized with periodic acid and a SAC spectrum run on the product; this spectrum also corresponded to that obtained from standard corticosterone similarly oxidized. Other faintly testing bands could not be identified (Fig. 5, Table 3). In Experiment HI14 dehydroepiandrosterone incubated with 644 mg. of adrenal slices failed to yield an extract with a 240 mμ absorption maximum.

TABLE 5. INCUBATION OF HUMAN INFANT ADRENAL GLANDS

Exp.	Weight of infant, grams	Sex	Age at death, hrs.	Interval between death and autopsy, hrs.	ACTH, IU./cc.	Chorionic gonadotrophin, I.U./cc.	UV*	FS*	PS*	TSC	Gas	Q_{O_2}
HI 1	350	F	13	2½	0.3	0	< 2				O_2	
HI 2	?	F	¾	1	0.3	0	< 2		<3		O_2	
HI 3	?	F	47	2	0.3	0	< 4	15		+	O_2	
HI 4	800	F	19	1½	0.5	0	<10		<2	0	O_2	
HI 5	750	F	18	¾	3.3	0	<36	20			air	1.6
HI 6	395	M	17	—	3.3	0	< 8	< 4			air	1.0
HI 7	610	F	55	4½	3.3	0	14	13		+	air	1.9
HI 8	1980	M	3½	2½	0	100	< 3	< 7			O_2	1.1
HI 9	450	F	2	1½	0	167	< 4	< 4			O_2	2.4
HI 10	750	F	1	2								
(twins)	575	F	1½	1½	0	100	< 3	< 3			O_2	1.3

* μg. 11-dehydro, 17-hydroxycorticosterone ⇔/100 mg. wet weight of adrenal slices.

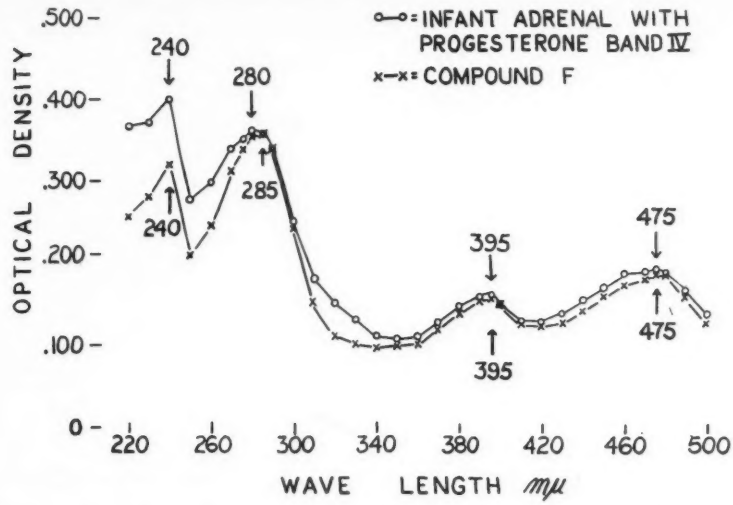

FIG. 5a. Human infant adrenals; incubated with progesterone; SAC spectra of Band IV (Fig. 2) and standard 17-hydroxycorticosterone (Kendall's compound F).

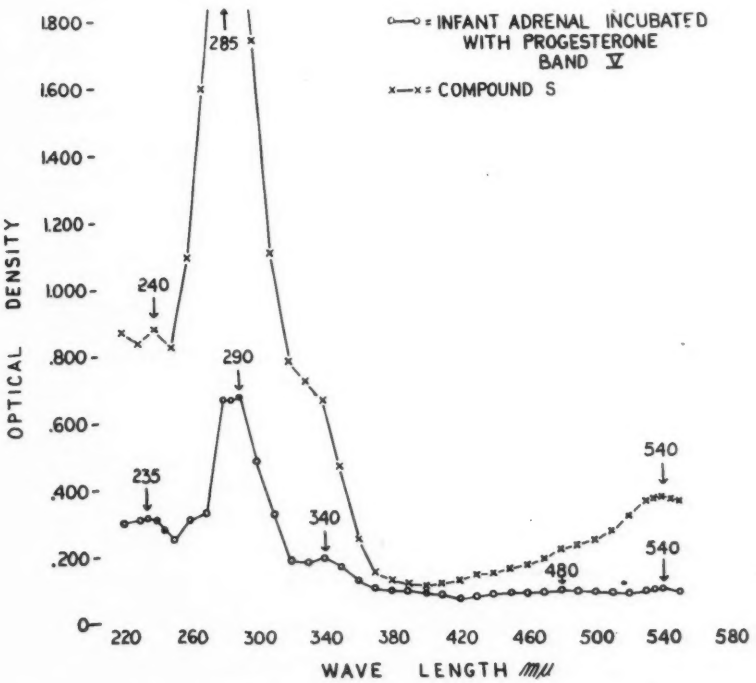

FIG. 5b. Human infant adrenals incubated with progesterone; SAC spectra of Band V (Fig. 2) and standard 11-desoxy, 17-hydroxycorticosterone (Kendall's compound S).

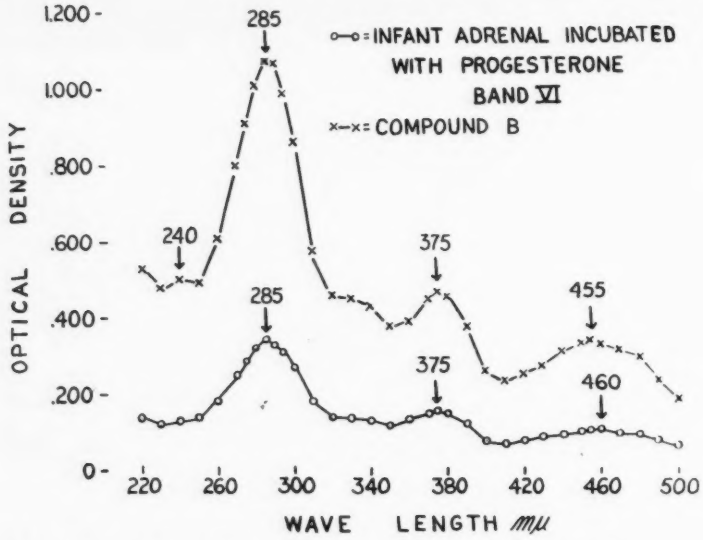

FIG. 5c. Human infant adrenals incubated with progesterone; SAC spectral of Band VI (Fig. 2) and standard corticosterone (Kendall's compound B).

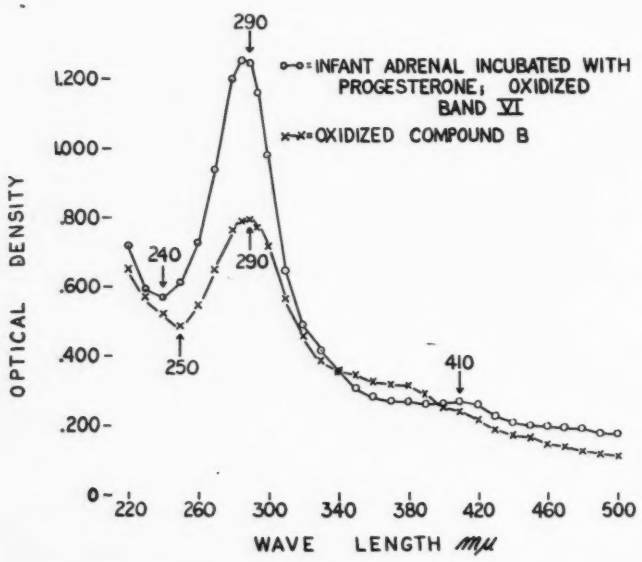

FIG. 5d. Human infant adrenals incubated with progesterone; SAC spectra of Band VI (Fig. 2) and standard corticosterone (Kendall's compound B), both after periodic acid oxidation.

DISCUSSION

Human adult and monkey glands on incubation repeatedly produced measurable amounts of steroid-like material by all the tests used, but human neonatal adrenal glands failed to produce such material consistently. However, with progesterone in the incubation medium, the infant adrenals produced PS chromogens and 17-hydroxycorticosterone and corticosterone and possibly 11-desoxy, 17-hydroxycorticosterone. Progesterone lies on the pathway of normal adrenal steroidogenesis (11). Its conversion to 17-hydroxycorticosterone requires directed hydroxylations at C-11, 17, and 21. These three reactions are characteristic of adult adrenal tissue, and the present study shows that the human neonatal adrenal was capable of carrying them out. We believe that the steroids found were formed from progesterone during the incubation and not merely extracted preformed from the adrenal tissue; several previous attempts with human infant glands incubated without added progesterone failed to yield sufficient SLM for satisfactory chromatograms, and in a previous experiment, tissue incubated with progesterone yielded PS material whereas adrenal tissue incubated without progesterone yielded none. The failure of the neonatal gland to synthesize steroid-like material without the addition of progesterone suggests that some earlier step in steroidogenesis was defective. In an effort to localize the block, the conversion dehydroepiandrosterone, a Δ^5, 3β-hydroxy compound, to a Δ^4, 3-ketone was attempted and failed. This system required the enzyme 3β-OH dehydrogenase. The human newborn adrenals were obtained postmortem, and the anoxia to which they were subjected may have destroyed this system if it was originally present. The adrenal gland of the premature infant is probably capable of complete steroidogenesis, since these infants present no unequivocal clinical evidence of adrenal insufficiency. The fetal adrenal might conceivably depend on the placenta for a supply of preformed progesterone, but other studies (12, 13) using bioassays indicate that the human fetal adrenal can produce material with progesterone activity, and Davis and Plotz (14) claim to have demonstrated cholesterol synthesis from C^{14}-acetate in human fetal adrenals. Bloch and coworkers (15) have tentatively identified 17-hydroxycorticosterone in extracts of fetal adrenals, though their method does not prove that the extracted steroids were formed by the gland. For example, Parker and Tenney (16) found considerable estrogenic activity in extracts of fetal adrenals, but no more than could be found in other fetal tissues. Gardner and Tice (17) have recently presented evidence that 17-hydroxycorticosterone and two unidentified unsaturated ketonic steroids were obtained by extraction of neonatal adrenals after prolonged alkali hydrolysis; they stressed the fact that no corticosterone was found in their studies. The present study indicates, however, that the neonatal gland is capable of forming corticosterone from progesterone *in vitro*.

Several attempts were made to find Zimmermann reacting chromogens

in human infant adrenal extracts. All failed except one; the successful attempt was made with the pooled extracts of 4 pairs of glands (total weight 3240 mg.) incubated in a tissue-culture medium. Small amounts of Zimmermann-reacting material (8 μg. per 100 mg. wet weight of tissue) were found in this instance. After chromic acid oxidation of an aliquot of this residue, no significant increase in chromogenicity occurred. The failure to demonstrate significantly increased chromogenicity in the Zimmermann reaction after chromic acid oxidation indicates the absence of steroids containing the 17-OH group. Many investigators (1) have suggested that the fetal zone makes androgens, many of which are 17-ketosteroids reacting in the Zimmermann assay. Recently, Bloch and coworkers (15, 18) reported the finding of minute amounts of three 17-ketosteroids in extracts from human fetal adrenals. However, the finding of 17-ketosteroids in Bloch's studies and of Zimmermann reacting material in the present study does not prove a significant androgenic function for the fetal zone. The amounts found were small and may have been formed in some other organ; similarly prepared extracts of other tissues might reveal similar findings.

The finding of 11-dehydro, 17-hydroxycorticosterone in an amount approximately equal to 17-hydroxycorticosterone after human adult adrenal incubation was unexpected. The patient was under cortisone therapy and a part of the 11-dehydro, 17-hydroxycorticosterone isolated may have been administered steroid which had been carried to the gland and merely extracted from the adrenal tissue or its contained blood.

SUMMARY

Adrenal glands were obtained from human premature infants dying shortly after birth and rapidly autopsied, from human adults undergoing adrenalectomy for prostatic carcinoma, and from sacrificed cynomolgus monkeys. The glands were sliced and incubated *in vitro* in various media.

Significant amounts of steroid-like material were repeatedly obtained from incubations of both adult human and monkey glands. From the former, 11-dehydro, 17-hydroxycorticosterone and 17-hydroxycortisone were identified and from the latter, 17-hydroxycorticosterone. From human neonatal glands a small amount of Zimmermann-reacting material was found after incubation in one experiment; otherwise steroid-like material was not regularly obtained without added steroid in the incubation medium.

With progesterone in the incubation medium, 17-hydroxycorticosterone, corticosterone, and possibly 11-desoxy, 17-hydroxycorticosterone were obtained after incubation of human neonatal adrenals, and 17-hydroxycorticosterone after incubation of monkey glands. These studies indicate the presence of three hydroxylating enzyme systems in human neonatal adrenal glands capable of introducing hydroxy groups at C-11, 17, and 21 of the steroid nucleus. These functions are characteristic of adult adrenal tissue.

Acknowledgments

The authors wish to acknowledge with thanks the help of Mr. Morris Silverman with tissue culture and of Mr. Richard Fairbanks, who performed the 17-ketogenic chromogen assay. Steroids for standards were kindly supplied by Ciba Pharmaceutical Co. and by the Schering Co. APL brand of chorionic gonadotrophin was kindly contributed by Ayerst, McKenna, and Harrison.

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SODIUM FLUOROACETATE DIABETES: INSULIN AND CARBOHYDRATE TOLERANCE AND THE ANTIKETOGENIC EFFECTS OF GLUCOSE AND FRUCTOSE

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THE locus of the metabolic defect in diabetes mellitus is still under debate, as is the site of action of insulin. The discovery that sodium fluoroacetate (SFA) precipitates a severe, but temporary, diabetes in the rat (1, 2, 3) offers an interesting opportunity in the study of the comparative biochemistry of diabetes since the basic metabolic disturbance due to SFA is reasonably well established. Fluoroacetate exerts its toxic effect only after its conversion by the tissues to fluorocitrate; the latter compound inhibits the enzyme aconitase in the Krebs cycle, resulting in an accumulation of citrate in the tissues (4). The temporary hyperglycemia and ketosis which ensues may be interpreted as the consequence of the Krebs cycle blockade with resulting loss of energy for phosphorylation of glucose, or its penetration into the cell, although the possibility of diminished insulin production by the beta cells as a consequence of the poisoning has not been ruled out (1, 2).

The present study was designed to characterize more completely the metabolic features of SFA diabetes with the objective of eventually comparing them to the better known alterations in alloxan and other types of experimental diabetes. Particular attention was directed to the response of the SFA diabetic rat to insulin and to carbohydrate loading and to the comparative antiketogenic effects of glucose and fructose. During the course of these studies it was established that SFA had a deleterious effect on the circulation which by itself modified the metabolic response.

METHODS AND MATERIALS

Male rats of the Vanderbilt strain weighing 200–300 gm. were used. Observations were generally made 4 or 16 to 24 hours after poisoning 24-hour fasted rats by intraperitoneal injection of 0.6 mg. SFA/100 gm. body weight. A freshly prepared 0.06% solution of SFA in normal saline was used. All procedures were performed on unanesthetized rats.

[1] Supported by a research grant from the American Cancer Society, administered by the Committee on Growth, the National Research Council and by Contract No. DA-49-007-MD-134, the Medical Research and Development Division, Office of the Surgeon General, Department of the Army.

Received July 25, 1956.

Blood sugar determinations were done by the method of Somogyi (5), blood fructose by difference after glucose oxidase incubation (6), blood ketones according to Werk *et al.* (7) and amino nitrogen by the technique of Frame, Russell and Wilhelmi (8). Blood pressure was estimated by the technique described in the report of Handler (9).

RESULTS

Glucose Tolerance. Tolerance to a glucose load by stomach tube (1 ml. of 20% glucose/100 gm. body weight) was tested 4 and 24 hours after SFA administration and compared to correspondingly fasted control rats (Fig. 1). In the latter there was no significant difference in glucose tolerance at 28 hours as compared to 48 hours of fasting. Four hours after SFA injection, the fasting blood sugar level was normal, but a striking and progressive rise occurred after the oral glucose administration, reaching a peak of over 350 mg.% in 3 hours. Twenty four hours after poisoning the fasting blood sugar level was markedly elevated and a further increase took place after glucose ingestion. These data confirm the severe degree of carbohydrate intolerance of the SFA poisoned rat previously reported (1).

Closer scrutiny of the tolerance curves of the SFA poisoned rats in Fig-

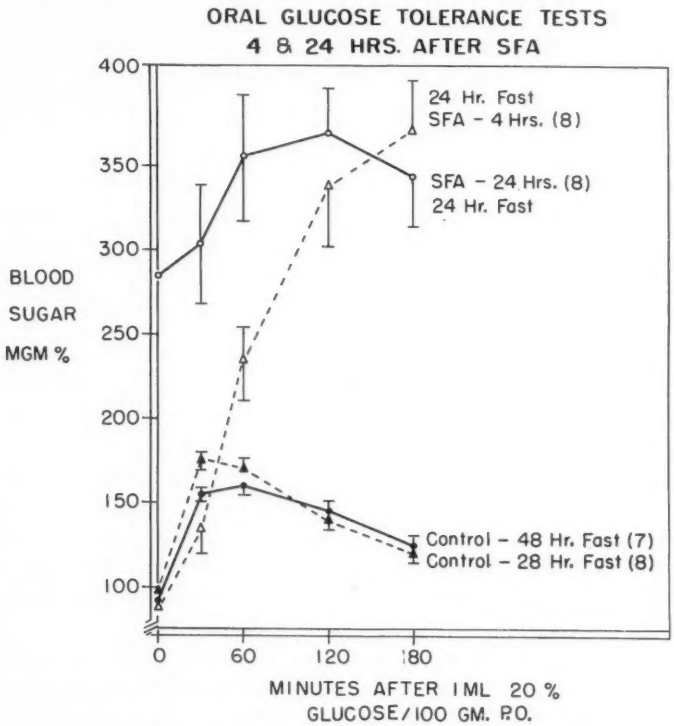

FIG. 1. Oral glucose tolerance test 4 and 24 hours after SFA compared to 28 and 48 hour fasted controls.

ure 1 revealed that despite their markedly diabetic character there was a significant delay in blood sugar rise during the first 30 minutes. This suggested that the poison might interfere with intestinal absorption of glucose, a not unexpected consequence of Krebs cycle blockade in the intestine. With this in mind, glucose tolerance tests were repeated by the intraperitoneal route four hours after poisoning and compared to the data from

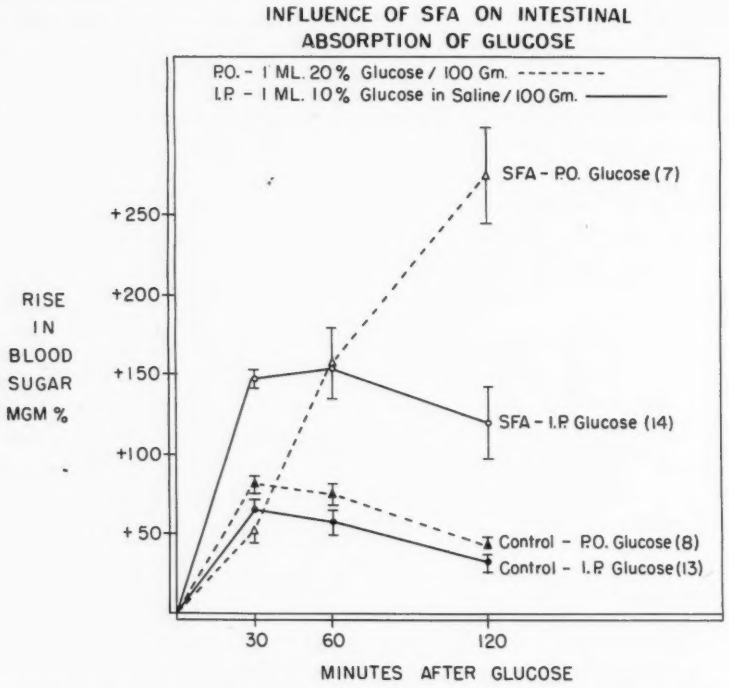

FIG. 2. Rise in blood sugar after oral and intraperitoneal glucose in control and SFA intoxicated rats. Note delayed rise in poisoned rats receiving glucose by mouth.

oral glucose administration. These results, recorded in Figure 2 as the mean rise in blood sugar after oral and intraperitoneal glucose in control and SFA poisoned rats respectively, demonstrate that there is no delay in blood sugar rise after intraperitoneal glucose as there is after oral glucose. Presumably SFA does in fact interfere with intestinal absorption of glucose.

Insulin Tolerance. The response to the intraperitoneal injection of 0.1 unit of glucagon-free insulin per 100 gm. body weight was tested 4 and 24 hours after SFA and compared to that of normal controls fasted 28 and 48 hours respectively. The data from the 4-hour experiment are plotted in Figure 3 and those from the 24-hour poisoned rats in Figure 4. In the first

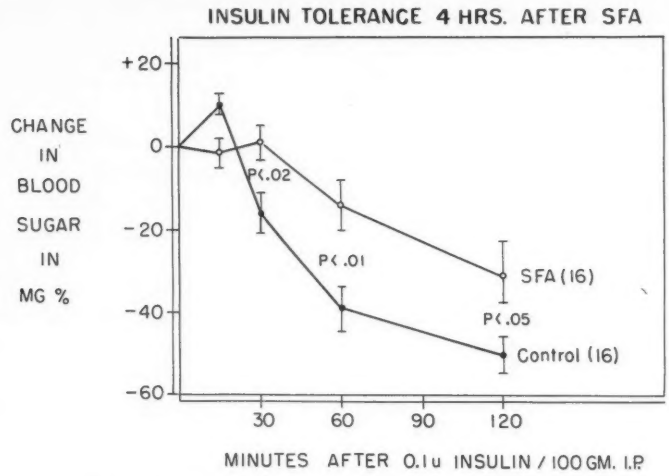

FIG. 3. Insulin tolerance 4 hours after SFA compared to that in the 28 hour fasted normal rats.

case a clear impairment in response to insulin in the poisoned rats can be detected since the blood sugar levels at the time of insulin administration were not significantly different, and hence the data could be plotted as absolute decrements from the starting point. At every point between 30 and 120 minutes, there was a significantly lesser fall in the blood sugar in the poisoned rats than in the controls.

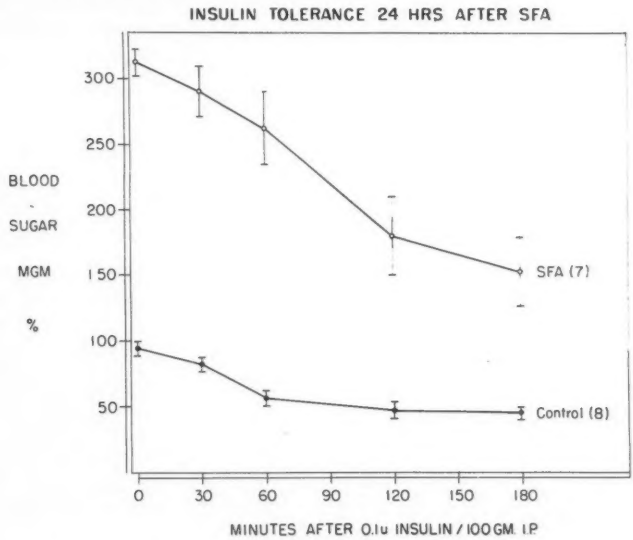

FIG. 4. Insulin tolerance 24 hours after SFA intoxication.

The insulin tolerance curve 24 hours after SFA (Fig. 4) is more difficult to interpret since the fasting blood sugar was 300 mg.% and it was not compared to a diabetic control. Considering the high initial blood sugar there would seem to be clear resistance to insulin in the SFA rats since the blood sugar fall during the first 30 minutes was the same as in the control. Moreover, recent data of Scow (Personal communication) show a fall of over 200 mg.% in the blood sugar of totally depancreatized rats following the subcutaneous injection of 0.03 u. insulin, i.e. approximately one-tenth the dose used here intraperitoneally.

The Antiketogenic Effects of Glucose and Fructose. There are data derived from both *in vivo* and *in vitro* studies indicating that the insulin deficient diabetic animal is capable of metabolizing fructose under circumstances where its ability to handle glucose is seriously limited (10, 11, 12). In particular, it has been claimed that fructose will ameliorate diabetic ketosis while glucose is relatively ineffectual (13, 14). This view, however, is not universally accepted (15). In light of these reports, it was considered worthwhile to investigate the effects of glucose and fructose on the ketosis of SFA diabetes: To this end the responses to the intraperitoneal injection of 1 ml./100 gm. of 0.9% saline, 10% glucose in 0.45% saline and 10% fructose in 0.45% saline respectively 4 and 16 hours after SFA were compared with those of normal control rats similarly treated. Total blood sugar and ketone levels were estimated at 0, 30, 60 and 120 minutes after injection.

Figure 5 shows that in this experiment even the saline injected rats showed a significant hyperglycemia 4 hours after SFA. In the poisoned rats the blood sugar rises after glucose and fructose were equally impressive. Since the technique for blood sugar analysis employed does not distinguish between glucose and fructose these data do not answer the question of whether the marked blood sugar elevations after fructose administration were due to fructose itself or to glucose derived from fructose in the liver. In order to settle this point, samples of blood from 5 normal and 4 SFA poisoned rats were treated with glucose oxidase according to the technique of Ashmore *et al.* (6) and the sugar content estimated again. The level of reducing material so determined during the fructose tolerance test can be assumed to be largely if not exclusively fructose since no reducing material could be detected in the blood after glucose oxidase treatment prior to fructose injection. The levels of fructose by this technique at 30, 60 and 120 minutes were 10 ± 1.86, 16 ± 1.75 and 5 ± 1.52 mg. per cent in the control rats and 22.5 ± 2.89, 24 ± 3.47 and 14.5 ± 1.50 mg. per cent in the SFA poisoned rats. From these data it can be assumed that fructose was taken up by the cells in the SFA poisoned rats and in part at least converted to glucose. The fructose levels in the SFA rats were slightly but significantly higher than those in the controls.

Evidence that the fructose was used metabolically by the liver along

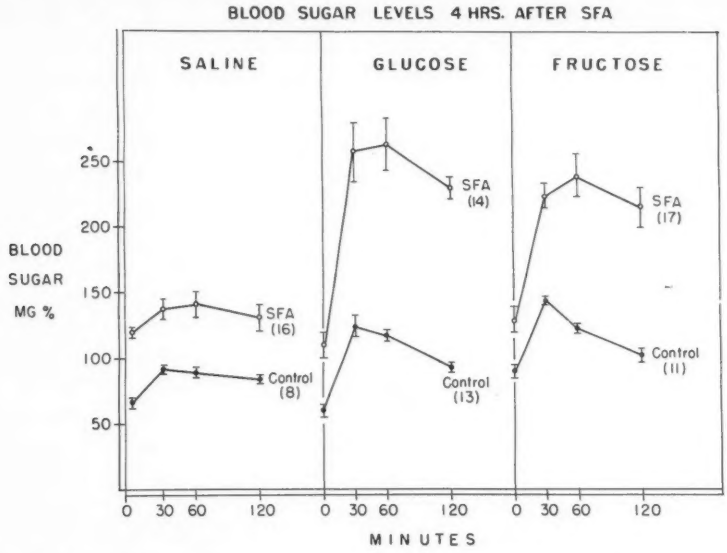

FIG. 5. Blood sugar levels after intraperitoneal injection of 1 ml./100 grams body weight of saline, glucose in saline and fructose in saline in normal fasted rats and in rats injected with SFA 4 hours previously.

paths other than conversion to glucose is found in the blood ketone data (Fig. 6). In the saline series there was an insignificant difference in blood ketone levels between the control and SFA treated rats, all showing a small increment in 2 hours. The control rats receiving either glucose or

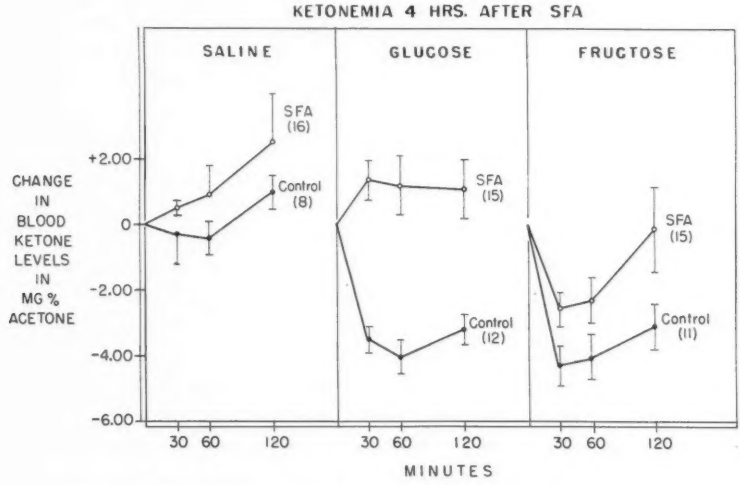

FIG. 6. Blood ketone levels in the rats of Fig. 5.

fructose exhibited a comparably sharp and significant decrement in ketonemia during the 2-hour period of observation. In contrast, the SFA poisoned rats showed no decrease in ketonemia at all after glucose and a response to fructose that was significantly less than the normal. Compared to glucose, however, fructose did have a definite and significant antiketogenic effect in the SFA poisoned rat. These data, coupled with the fructose levels noted above, establish that the SFA poisoned rat is quite capable of utilizing fructose, even though it be at a lesser rate than normal.

When the comparable study was attempted 16–20 hours after poisoning, difficulty was encountered because preliminary studies revealed a tendency for blood ketone levels to decline regardless of whether saline, glucose or fructose was administered. Indeed, it was found that even with no treatment at all ketonemia tended to decrease in successive blood samples. This observation was most disconcerting until careful observation of the clinical state of the poisoned rats suggested that some of the animals were suffering from peripheral circulatory collapse, with a propensity to succumb shortly after the tolerance tests. It was noted that handling of the rats and bleeding from the tail frequently resulted in a rapid deterioration in the clinical appearance of the animals. Since we had previously shown that peripheral circulatory collapse itself will lower blood ketone levels (16), it seemed altogether likely that the blood ketone changes noted in these SFA poisoned rats might be accounted for on a similar basis.

With this in mind all previous data on saline, glucose and fructose injections 16–20 hours after SFA were discarded and the experiment repeated in such a way as to give some indication of the possible interfering role of peripheral circulatory failure. This was done by estimating blood amino nitrogen in all animals since it has been known for sometime that the blood amino nitrogen level rises during shock in the rat (17, 18). While no previous information was available on the effects of SFA on blood amino nitrogen levels, it was thought conceivable that a classification of rats in terms of degree of circulatory failure might be feasible from this criterion. To this end blood amino nitrogen levels were estimated in control rats receiving either saline, glucose or fructose intraperitoneally. The mean blood amino N level plus 2 standard deviations was calculated for each point on the curve. Blood amino N levels were likewise determined in SFA poisoned rats. Any rat from the latter series which had a single blood amino N value exceeding the corresponding control by more than 2 standard deviations was arbitrarily classified as in shock while those animals in which the amino N level remained within 2 standard deviations of the control throughout the entire curve were considered not to be in shock. As seen in Figure 7, a fairly discrete separation could be made among the SFA poisoned rats by this arbitrary classification, although in the glucose series the separation was distinct only at the 60 and 120 minute points.

As a further corroboration of the clinical impression of peripheral cir-

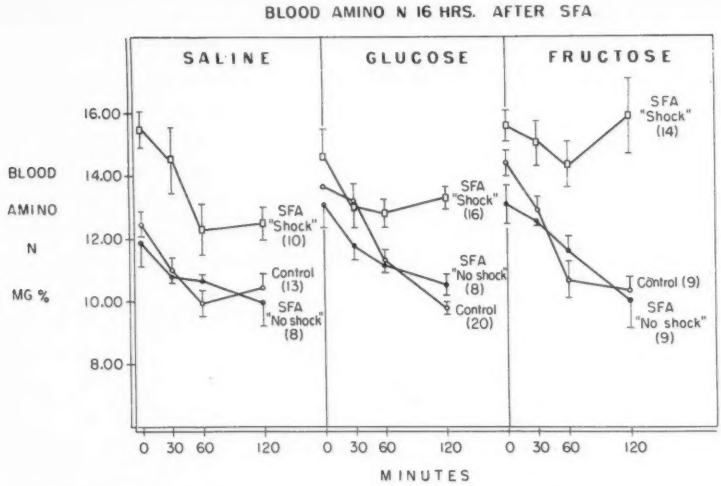

FIG. 7. Blood amino nitrogen levels in rats poisoned with SFA 16 hours previously and controls similarly fasted. See text for basis for classification into "shock" and "no shock" groups. Tolerance tests as in Figures 5 and 6.

culatory failure the blood pressure was measured in the tails of a number of normal and SFA poisoned rats at the end of the tolerance test and these were plotted against the change in blood amino N during the test. Figure 8 shows that most poisoned rats exhibited some degree of hypotension and that in general there was a tendency for the hypotensive rats not to show

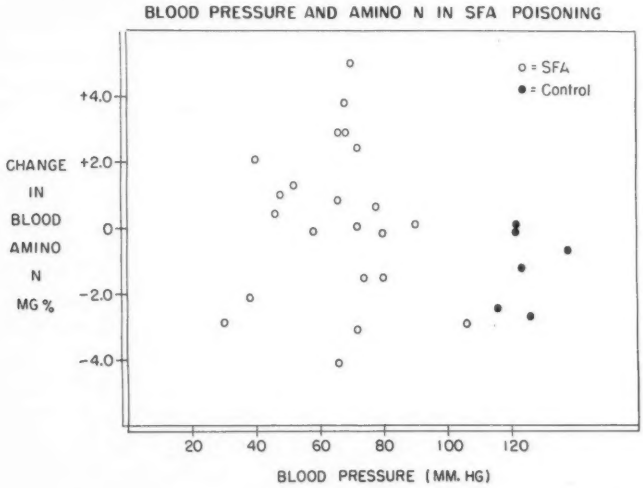

FIG. 8. Tail blood pressure levels in mm. Hg. at the end of tolerance tests plotted against the change in blood amino N levels in control and SFA poisoned rats.

falling blood amino N levels as did the controls. Several poisoned rats exhibited marked hypotension and yet had normal falls in blood amino N. These exceptions might be anticipated in any experiment in which only sporadic blood pressure determinations were made since earlier work showed that hypotension must be sustained for some time before hyperaminoacidemia develops (17).

Returning to the ketone studies it was found that when the blood ketone values for the poisoned rats were plotted together, irrespective of blood amino nitrogen levels, all groups showed a fall in blood ketone levels re-

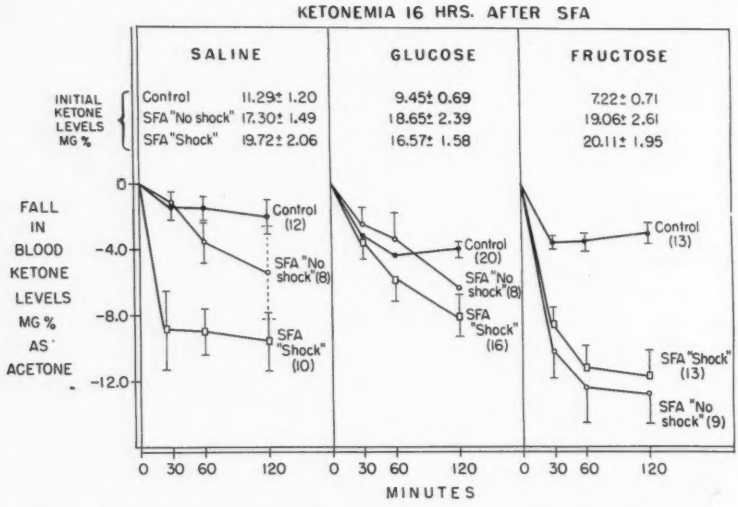

FIG. 9. Blood ketone levels plotted as fall in mg. percent during saline, glucose and fructose tolerance tests 16 hours after SFA. The data from the poisoned rats are subdivided into "shock" and "no shock" groups according to the blood amino N criteria described in text.

gardless of treatment, as noted in the preliminary studies. In contrast, when the data are broken down in terms of the amino nitrogen values into "shock" and "no shock" groups a difference in response to glucose and fructose is discernible (Fig. 9) as it was in the four hour experiments (Fig. 6). In the latter, peripheral circulatory collapse did not appear to be a significant problem. This was confirmed in a series of animals in which the blood amino nitrogen concentrations were estimated and no separation into two groups could be made as in the 16 hour experiments. To conserve space these data are not recorded.

In Figure 9 the ketone curves are plotted as decrements from the starting values. The initial ketone values are recorded at the top of the figure. The absolute decrements in the poisoned animals are harder to evaluate than

in the 4-hour experiment since the blood ketone levels are so much higher than in the controls. In the saline series, the "shock" group show a significantly greater fall than do the "no shock" or control groups. In the glucose series the difference between "shock" and "no shock" is not clear-cut, but it is noteworthy that the fall in blood ketones in the "no shock" group is not significantly different from the corresponding group which received saline. This contrasts with the situation when the animals receive fructose. Here there is a marked and significant fall in blood ketones, even in the absence of shock, when compared to the glucose and saline groups. The

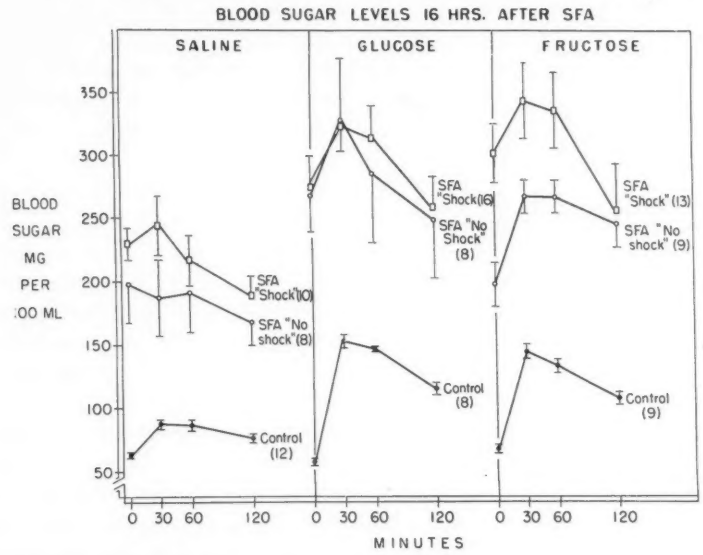

FIG. 10. Blood sugar levels in 16 hours SFA poisoned rats during saline, glucose and fructose tolerance tests.

animals which are in shock according to the amino N criteria also show a marked drop in ketones, presumably as a consequence of the circulatory changes as well as the fructose. It is apparent from these data that despite the complications resulting from the circulatory failure a difference in the antiketogenic response to fructose and glucose is discernible in the SFA poisoned rats.

Figure 10 plots the blood sugar values in the same animals. There was a tendency for the carbohydrate tolerance curves to show greater impairment in the rats classified as in shock, but this was valid statistically only in the fructose series. Since the initial ketone levels before handling the rats were higher in the "shock" group this suggests that a more severe degree of of diabetes predisposes to peripheral circulatory failure, rather than vice versa, i.e. that shock accentuates the carbohydrate defect.

DISCUSSION

The data recorded in this report confirm and extend those previously published from this laboratory (1, 2). A significant degree of carbohydrate intolerance is detectable within 4 hours of poisoning the rat with SFA and frank fasting hyperglycemia is apparent in 16–24 hours. At the same time there is a definite resistance to the blood sugar lowering effect of insulin.

This diabetogenic response to SFA with an associated insulin insensitivity is the anticipated response to an agent such as SFA, the main action of which is presumed to be the blockade of the Krebs cycle. The tricarboxylic acid cycle is of particular importance in the generation of high energy phosphate bonds and hence in the maintenance of the stores of ATP necessary for the phosphorylation of glucose via the hexokinase reaction as well as for other energy requiring processes, including possibly glucose transport into the cell. Impaired glucose utilization would thus be an expected consequence and was indeed found. This impairment of glucose utilization is comparable to that in other types of diabetes but seems to differ in at least one important respect, namely in being demonstrable in the gut as an apparent defect in glucose absorption. A similar disturbance in intestinal absorption is not a feature of uncomplicated diabetes mellitus. Regardless of whether the intestinal absorption of glucose involves phosphorylation as the first step or whether some other transport mechanism is responsible, it is apparent that this process is not limiting in the intestine in diabetes mellitus as it is in SFA poisoning. This points up one important difference between the diabetes of insulin deficiency and that of fluoroacetate intoxication.

In addition to glucose intolerance there is also some degree of insulin insensitivity. Short of a near complete block in the Krebs cycle, which would be rapidly fatal, one would expect that the response to insulin in the SFA diabetic rat would be blunted but not abolished. It has already been demonstrated (1, 19) that metabolites which may serve as intermediaries for the Krebs cycle, or precursors thereof, may act as antidotes for SFA poisoning by supplying enough substrate to bypass the block. Insulin might increase the efficiency of glucose transport and/or phosphorylation, making available more substrate for the Krebs cycle, but in the final analysis the total availability of high energy phosphate generated by the cycle might still be a limiting factor in glucose utilization. The simultaneous administration of insulin and glucose has been suggested in the treatment of fluoroacetate intoxication (19).

The data clearly indicate that fructose is readily metabolized in the fluoroacetate poisoned rat. Not only is it converted to glucose but presumably it is metabolized in the liver in such a manner as to inhibit ketogenesis. To do so it must allow for the metabolism of acetyl coenzyme A either via the Krebs cycle or for fat synthesis, thereby cutting down the accumulation of acetoacetyl Co-A in the liver. In this regard it might be noted that the formation of ketone bodies by the liver might be as char-

acteristic for this tissue as is citrate accumulation in other tissues and may in turn account for the fact that citrate does not ordinarily reach very high levels in the liver of the SFA poisoned animal (20). A block at the level of aconitase would be expected to result in an accumulation of citrate as well as a backing up and accumulation of compounds behind citrate. In the case of acetyl-Co A this can readily drain from the liver as acetoacetate thereby tending to keep down the concentration of citrate. In other tissues the enzymatic mechanisms do not favor acetoacetate formation and release.

The relatively rapid metabolism of fructose compared to glucose in the SFA poisoned rat may be related in part to the known greater activity of fructokinase as compared to glucokinase (12, 21, 22, 23). Our data point to some diminution in fructose utilization in the poisoned animals compared to normals, but it is apparent that the loss of energy from the Krebs cycle in the poisoned rats has much less impact on fructose than on glucose utilization in the liver. It is not immediately apparent whether the rate of provision of high energy phosphate is the limiting factor in hepatic glucose utilization; it certainly is not in the case of fructose and yet the amount of high energy phosphate available for both processes in the poisoned rat must be equivalent. An entirely acceptable explanation for the antiketogenic action of fructose as compared to glucose is not now apparent. It might relate to the fact that fructose enters the glycolytic system at the triose stage (6, 21) and thereby is more immediately available than glucose.

The discovery that SFA induces peripheral circulatory failure is intriguing and has interesting implications for the shock problem. These are discussed elsewhere (24). The development of variable and unpredictable degrees of peripheral circulatory failure on handling or bleeding the late SFA poisoned animals poses some difficulties in experimental design and interpretation when one is primarily interested in the diabetes. The estimation of the metabolic contribution from the circulatory derangements by measurements of blood amino nitrogen level diminishes but does not obviate the difficulties. This factor must be kept in mind in the interpretation of all past and future work with sodium fluoroacetate in the intact animal. With reasonable care and some understanding of its metabolic consequences, peripheral circulatory failure need not be a deterrent to further studies on SFA diabetes.

SUMMARY

Four and 24 hours after the intraperitoneal injection of sodium fluoroacetate (SFA), there is marked impairment of glucose tolerance and significant resistance to the hypoglycemic action of insulin. The oral glucose tolerance curve shows a delay in the absorption of glucose in the SFA treated rat compared to the normal and to the SFA poisoned rat receiving glucose intraperitoneally.

Total blood sugar and ketone levels were estimated before and after

intraperitoneal injection of saline, glucose or fructose into rats 4 and 16 hours after SFA and compared to normal controls.

Four hours after SFA blood sugar levels were significantly elevated after saline and markedly so after glucose and fructose. Blood fructose levels rose only slightly indicating that the high blood sugar values in the SFA poisoned rats represented conversion of fructose to glucose. Fructose levels were slightly but significantly higher in the poisoned animals.

Both glucose and fructose exerted comparable antiketogenic effects in normal fasted rats, whereas only fructose was antiketogenic in the 4 hour SFA poisoned rats. However, the antiketogenic effect of fructose was significantly less than normal in the poisoned animals.

Sixteen to 24 hours after SFA there was marked hyperglycemia with further striking blood sugar increase after glucose or fructose. Blood ketone levels, however, were unstable, tending to fall spontaneously from their initially high values. This fall was shown to be due to the ready development of peripheral circulatory failure on handling the poisoned animals.

By classifying the rats into "no shock" and "shock" groups in terms of the behavior of the blood amino nitrogen levels during saline, glucose or fructose infusion, it could be shown that when the antiketogenic effect of circulatory failure was minimal, glucose was relatively ineffectual whereas fructose exerted a definite antiketogenic effect.

The significance of these results is discussed in terms of the comparative metabolic defects of SFA and pancreatic diabetes.

ADDENDUM

Since completion of this manuscript the influence of SFA on alloxan diabetic rats has been studied. The rats had received alloxan at least 4 weeks before being poisoned with SFA and were only mildly diabetic. SFA administered at the end of a 24 hour fast caused a marked increase in glycemia and ketonemia 4 and 24 hours after injection. As no further studies are contemplated with the alloxan diabetic rat, the data are herewith recorded. The blood glucose and ketone levels before SFA injection and 4 and 24 hours later respectively were glucose 75 ± 5.3, 103 ± 5.9 and 243 ± 8.1 mg. per cent and ketones 5.74 ± 0.61, 7.23 ± 1.46, and 14.27 ± 2.42 mg. per cent in the 12 SFA treated controls and 124 ± 12.1, 332 ± 33.9 and 404 ± 39.8 mg. per cent and 3.01 ± 0.32, 18.70 ± 2.61 and 41.28 ± 5.34 mg. per cent in the 15 SFA-treated alloxanized rats.

Acknowledgments

We are indebted to the Mead Johnson Co., Evansville, Indiana for generous supplies of 10% fructose as Levugen, and the Eli Lilly Co., Indianapolis, Indiana for the glucagon-free insulin.

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COMPARATIVE HISTOLOGICAL STUDY OF THE ADRENAL CORTEX OF THE WILD AND DOMESTICATED NORWAY RAT[1]

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THE domestication of the wild Norway rat into the tame laboratory rat has produced striking alterations in temperament, anatomy and physiology (1). Because domestication involves obvious changes in the psychology of the animal the neuroendocrine system has received a large share of the emphasis in past studies which have sought to elucidate the role physiologic mechanisms play in domestication.

The adrenals have long been known to show important differences between the wild and tame Norway rat. Watson reported in 1907 that the adrenals of the domesticated rat are smaller than those of the wild rat (2). King and H. H. Donaldson (3) and King (4) later found that in successive generations of wild rats bred in the laboratory the adrenals gradually become lighter and tend to approach the weights found in domesticated rats. J. C. Donaldson showed that the adrenal cortex accounts for the entire difference in size (5). This has been confirmed by Rogers and Richter (6).

This report contains the results of a study made on the adrenal cortex in these two strains of rats to determine whether histochemical differences could be demonstrated. The study was carried out over a period of several years on wild Norway rats shot in the field or in the laboratory after various periods of captivity, and on domesticated Norway rats maintained from birth under ideal laboratory conditions.

METHODS

Over 100 domesticated rats and 76 wild Norway rats were used. Eleven domesticated rats were killed specifically for this study by shooting through the chest. The remainder had served as controls for other experiments and were re-used for this study provided there were no alterations in environmental conditions.

The domesticated rats came from the Richter colony, which has been in continuous existence for over 30 years, having been started with albino rats from the Wistar Insti-

Received August 21, 1956.

[1] Carried on in part under a contract between the Department of the Navy, Office of the Naval Research Biology Branch, and the Johns Hopkins University; and in part under a grant between the U. S. Public Health Service and the Johns Hopkins University.

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tute. A few non-albinos from the stock of Dr. E. V. McCollum were added about 25 years ago. During a four year period (12–16th year) of the colony's existence some rats, especially females, were selected for running activity. There has been no other planned selection. From the time of weaning the rats were fed a standard diet[3] and tap water *ad lib*, with a weekly supplement of lettuce. They were kept in a well ventilated room at an average temperature of 77° F and all were handled at least once a week.

The 76 wild Norway rats may be divided into 2 groups: (A) 26 adult rats shot on a farm near Baltimore where no efforts at rat control had been made for many years, and (B) 50 adult rats trapped in the alleys and backyards of Baltimore. The farm rats had access at all times to ample amounts of grain in and around horse stalls and to large amounts of water once or twice a day when buckets were placed in stalls. At other times they satisfied their thirst with rain water or horse's urine. Block salt was usually present in the feeding troughs. The chief environmental hazards were barn owls and a few dogs. The city rats were brought into the laboratory within 24 hours after trapping, and were kept for varying lengths of time before killing. In captivity the wild rats were given free access to Purina lab chow and tap water.

All rats were killed by shooting, by crushing the cervical cord with pliers, or by a blow on the head. The adrenals were promptly removed, cleaned of periadrenal fat, weighed on a torsion balance and fixed without delay in 10% neutral formalin. The method of killing made no apparent difference under these conditions.

A half or whole adrenal from each rat was dehydrated, embedded in paraffin, sectioned at 6 or 7 μ and stained with Harris hematoxylin and eosin Y. The other half or whole adrenal was usually fixed two or more days and then sectioned at 15 μ on the freezing microtome. The plane of the section passed transversely as near as possible to the center of the gland. The frozen sections were stained with sudan IV, sudan black B, the Schiff leucofuchsin reagent, Mallory's modification of the Schultz sterol method (7), and the modified Ashbel-Seligman method for lipoidal ketonic carbonyl groups (8). Some of the glands were washed over night before sectioning; in other cases the individual sections were washed in distilled water for 15 to 30 minutes. This made no difference in the results with the exception that over night washing was necessary for the Ashbel-Seligman reaction. Most frozen sections were mounted on gelatin or albumen-coated slides and stained on the slides. A few were stained before mounting. Cover slips were mounted with glycerin-jelly.

The sudan stains were chosen to demonstrate lipids in general. Sudan black B was found to be too intense and to have too great an affinity for other intracellular constituents to be of value in this study. Therefore, more attention was centered on the Sudan IV stained material. The Schultz cholesterol test agreed closely in its results with the Sudan IV and was used only on occasional sections. The Schiff and Ashbel-Seligman tests were used in parallel with the sudan stain because they give color reactions with aldehydes produced by autoxidation of highly unsaturated lipid during fixation and washing, and in the case of the latter, possibly other carbonyl groups from steroids, or steroid precursors (9, 10).

The photographs used to demonstrate differences in staining reactions were standardized as much as possible by using the same film and processing lots, and by standardizing exposures, light and aperture settings as well as time in the various developing and fixing solutions. Exposure of printing paper was electrically controlled by a timed light switch. All films and prints of frozen sections shown in this report were simultaneously processed in the same solutions.

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[3] Graham flour 72.5%, casein 10.0%, skim milk powder 10.0%, butter 5.0%, calcium carbonate 1.5%, and sodium chloride 1.0%.

RESULTS

Gross Anatomical Differences

The wild rat adrenals were brownish in color and pyramidal in shape. They were much larger than the domesticated rat adrenals which were pinkish-tan in color and spherical in shape. The relative size of the adrenals in these two strains is shown in Figures 1 and 2.

Histochemical Differences

Capsule.—The capsule in the wild rat gland averaged 10 μ in thickness as compared with an average of 14 μ in the domesticated rat. It was composed of compact fibrous tissue with scattered cells containing long narrow nuclei and scanty cytoplasm; there was generally a sharp distinction between the capsule and the glomerulosa (Fig. 3). The capsule of the domesticated rat adrenal had a looser arrangement with occasional cells containing small sudanophilic droplets present in the connective tissue lattice-work. In some areas there was no sharp distinction between the capsule and glomerulosa (Fig. 4).

Glomerulosa Zone.—This zone of the wild rat adrenal was made up of cells which were quite large (Fig. 3), and were heavily packed with sudanophilic droplets (Figs. 6, 7, 9). There was an intense reaction with both the Schiff (Figs. 10, 12) and the Ashbel-Seligman reagent. The orientation of cell cords about the capillaries was not as tortuous as in the domesticated rat.

The glomerulosa cells of the domesticated rat adrenal were smaller. (Fig. 4). The lipid was quite sparse and arranged in a patchy fashion in some sections (Figs. 13, 14, 15, 16, 17, 19). The Schiff and Ashbel-Seligman reactions usually reflected the staining density of the sudan stain in this zone (Figs. 18, 20).

Transition Zone.—Most wild rat adrenals showed only a faint trace of this sudan-negative zone between the glomerulosa and fasciculata zones (Figs. 7, 8, 9, 11), and some lacked it altogether. In a few wild rats a heavy layer of lipid appeared in place of the usually lipid-free transition zone (Fig. 5).

A transition zone was always present in the domesticated rats and was usually much wider than in the wild rats (Figs. 13, 14, 15, 16).

Fasciculata Zone.—The fasciculata zone showed some of the greatest contrasts between the two strains. In the wild rat the outer half of the zone usually stained heavily with the sudan stains and Schiff and Ashbel-Seligman reagents and gave a strongly positive Schultz sterol reaction (Figs. 5, 6, 7, 8, 9, 10, 12). In the inner half of the zone, there was usually a decreased amount of lipid (Figs. 6, 8, 9, 11). Throughout the fasciculata in the wild rat the capillaries were prominent and often congested with blood.

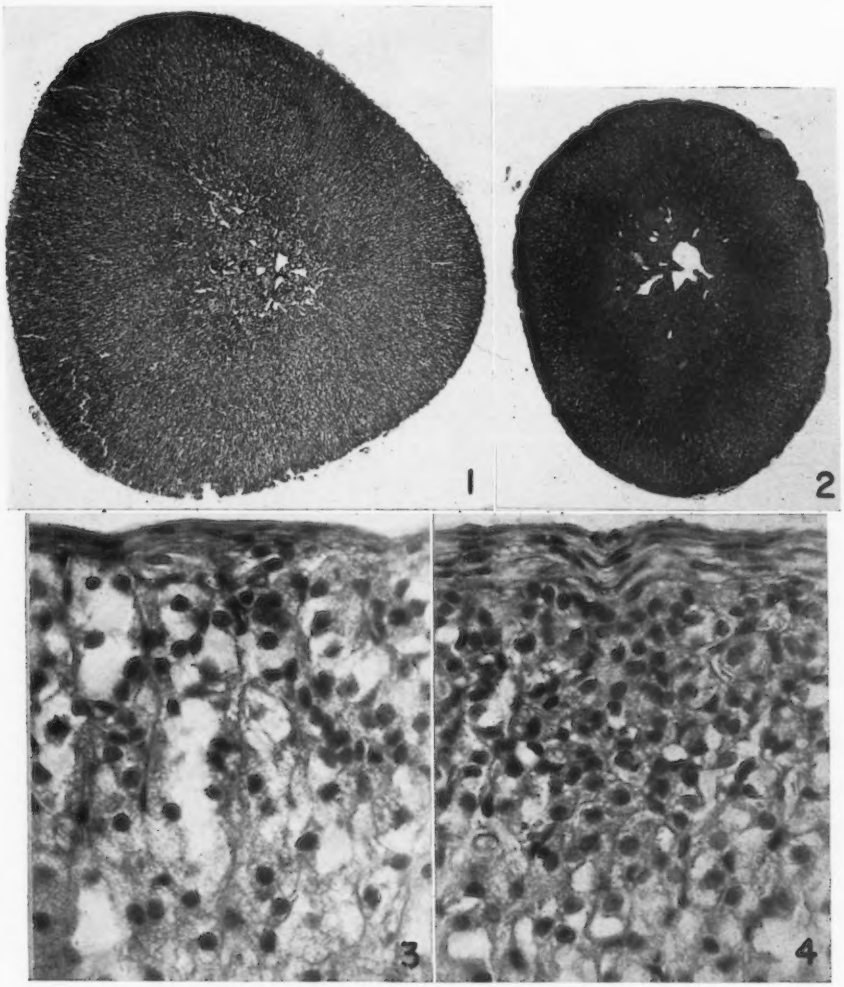
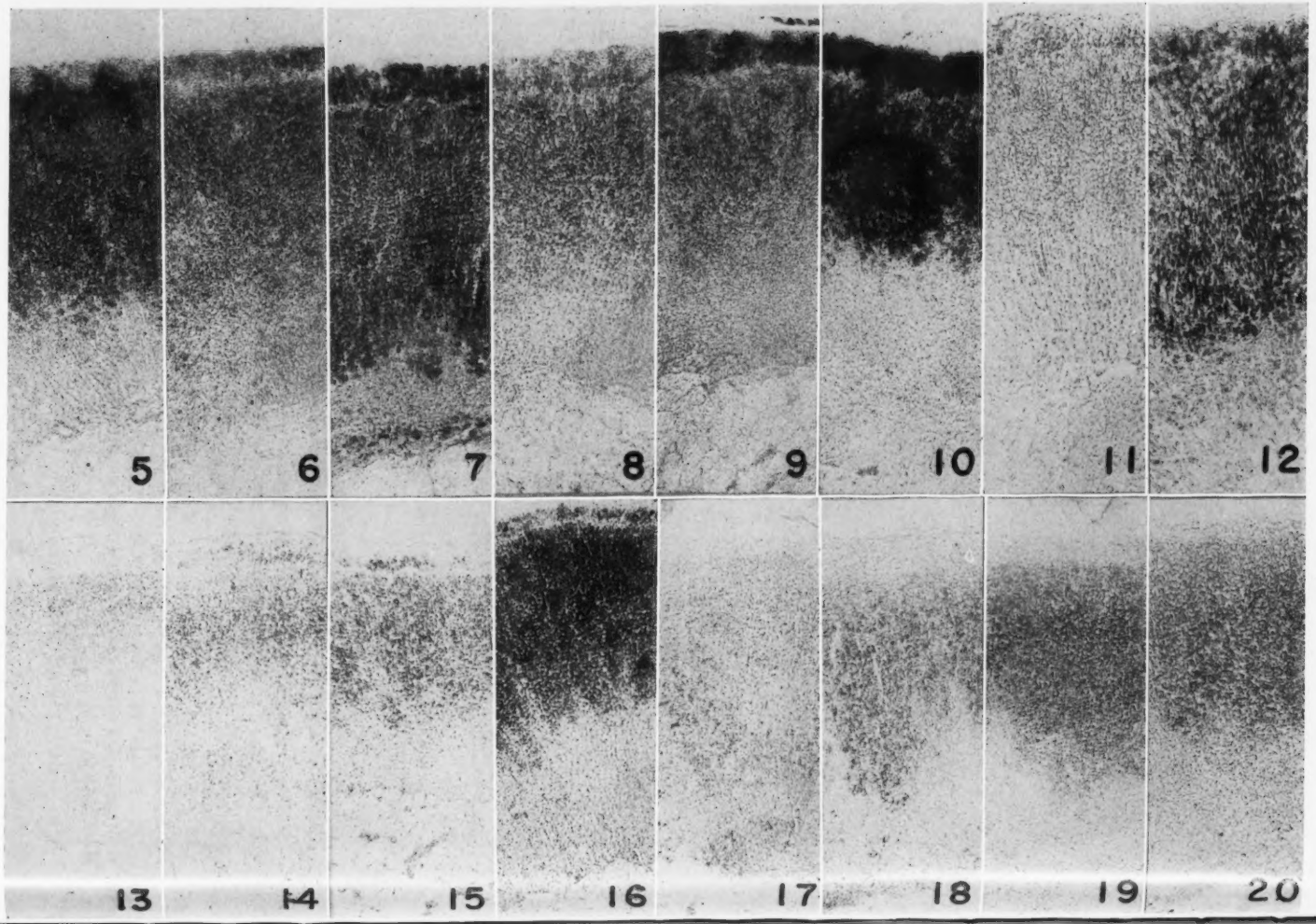


FIG. 1. Transverse section through adrenal of wild Norway male shot on farm: body weight 395 gm. The combined adrenals weighed 156.0 mg. H&E, ×25.

FIG. 2. Transverse section through adrenal of domesticated rat; body weight 388 gm. The combined adrenals weighed 38.5 mg. H&E, ×25.

FIG. 3. Highly magnified outer cortex of wild rat adrenal showing thin capsule and vacuolated glomerulosa cells. H&E, ×500.

FIG. 4. Same magnification as in Figure 3 in outer cortex of domesticated rat adrenal showing thicker capsule and small, densely packed glomerulosa cells with small vacuoles. H&E, ×500.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In the domesticated rat the fasciculata zone contained much less lipid and active aldehyde and ketonic carbonyl groups (Figs. 13, 14, 15, 17, 18, 19, 20), however, an occasional rat showed a moderately heavy concentration (Fig. 16). In some areas the lipid had a patchy distribution or was lacking altogether. The capillaries were not prominent in the domesticated rat.

Reticularis Zone.—The wild rat reticularis has widely dilated sinusoidal capillaries separating the cell cords. In some specimens these capillaries were empty; others showed extreme congestion. There was usually a slight amount of lipid present in the reticularis.

The domesticated reticularis zone had inconspicuous vascular channels and its cell cords were more tortuous than in the wild rat. There seldom was appreciable lipid present in this zone in the domesticated rat.

In both strains occasional cells bordering the medulla contained small or large droplets staining with sudan IV, and the Ashbel-Seligman and Schiff reagents (Fig. 7).

Medulla.—The medulla cells of the domesticated rats were usually somewhat foamy in appearance with small vacuoles (Fig. 21), whereas, in the wild rats the medulla cells were often distended with large vacuoles (Fig. 22). Both strains had occasional collections of ganglion cells. The blood sinusoids appeared to be proportionate to gland size. In the interstices of the wild rat medulla there were often a few scattered polymorphonuclear eosinophils. These were not seen in the normal domesticated rat medullas.

Fetal (Provisional) Zone.—There was no persistence of fetal or provisional zone in any of the domesticated rats examined nor among the wild rats killed on the farm. In two of the adult wild rats which had been killed in the city of Baltimore, there were the provisional type of cortical tissue made up of large, compact eosinophilic cells with rather dark nuclei, and located between the reticularis and medulla. One of these showed large islands of these cells scattered through the medulla.

FIGS. 5, 6, 7, 8, 9, 11. Sudan IV stained frozen sections of normal wild Norway adrenals showing individual variations. In Figure 5, note the dense staining in place of a transition zone. Most wild rat sections have a dense collection of lipid in the glomerulosa zone and a faint transition zone. All ×30.

FIG. 10, 12. Schiff stained sections of the same adrenals represented in 9 and 11, respectively. Note the denser appearance of the Schiff stain as compared with the sudan IV in the same rat. All ×30.

FIGS. 13, 14, 15, 16, 17, 19. Sudan IV stained frozen sections of normal domesticated adrenals. Note great variability in amount of lipid present in the cortex. There is a transition zone present in all the sections. All ×80.

FIGS. 18, 20. Schiff stained sections of the same adrenals represented in 17 and 19, respectively. Note that the density of the Schiff stain appears same as that of the sudan IV stained section. ×80.

The Effect of Urban Environment and Captivity

Rats captured or shot in the city of Baltimore showed more lipid and material reacting with the Schiff and Ashbel-Seligman reagents in the adrenal cortex then were seen in the farm rats. The increase was chiefly in the inner half of the cortex and tended to produce a uniform lipid density from the capsule to the medulla. The transition zone was usually absent. There was more variation noted in the specimens of city rats than those of the farm rats. A few of these glands appeared histologically quite similar to the farm rat glands.

About half of the wild rats shot on the farm had colloid droplets in the

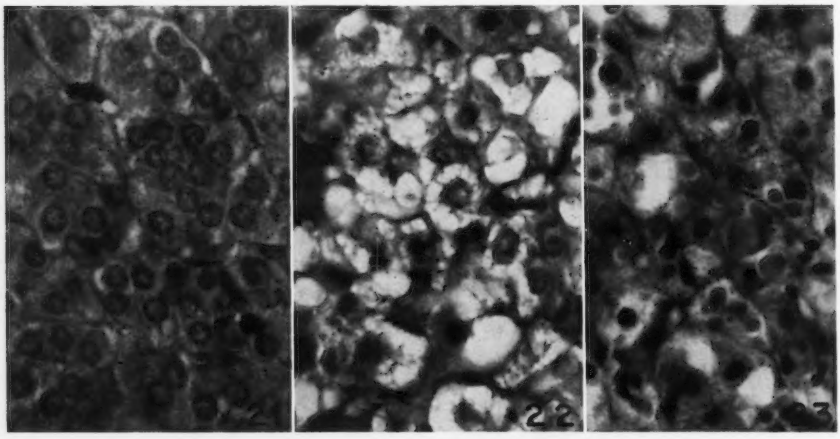

FIG. 21. Normal domesticated rat medulla showing relatively little vacuolation. H&E, ×560.

FIG. 22. Normal wild rat medulla showing numerous large vacuoles. H&E, ×560.

FIG. 23. Highly magnified area in the outer fasciculata zone of a wild Norway rat shot on a farm showing large colloid accumulations in the cells. H&E, ×560.

fasciculata and, to a lesser extent, in the reticularis; while no colloid was seen in the adrenals from the wild city rats or the domesticated rats. An extreme example of fasciculata colloid is shown in Figure 23.

After captivity of several days to months there were increased amounts of lipid, aldehyde and ketonic carbonyl groups in all regions of the cortex.

DISCUSSION

Practically all the differences between the adrenals of the wild and domesticated Norway rat in this study are quantitative. One sees in the wild rat not only a larger gland but a heavier concentration of lipid, aldehyde, ketonic carbonyl groups, and a richer blood supply. These facts favor the interpretation that the wild rat adrenal cortex may be in a state of heightened secretory activity.

It is curious that in the wild rat the increased size, lipid content and

vascularity is present in all the three major anatomic zones since in the rat, at least, there is ample evidence that the different adrenal zones are not only anatomically distinct but functionally specific. The glomerulosa zone of the rat is the zone most affected by experimental alteration of body electrolytes, principally sodium and potassium, and the fasciculata zone most strikingly affected by hypophysectomy or ACTH stimulation (11).

One should not expect, therefore, that the same factors are responsible for the increased size and lipid content of each adrenal zone. In the case of the wild Norway rat there are well known environmental factors which can account separately for increased cortical activity in the glomerulosa zone on the one hand and the fasciculata and reticularis zones on the other.

The wild rat's usual diet in nature is low in salt (12). Thus, the increased volume and lipid content of the wild rat glomerulosa zone may be a compensatory hyperplasia to produce an optimum sodium balance in the face of a low sodium intake. A low sodium intake produces an increase in sudanophilic lipid as well as hypertrophy of the glomerulosa zone of the domesticated rat (13). Because of this compensatory hyperplasia, freshly trapped rats would be expected to retain excess sodium when fed laboratory chow containing greater amounts of salt until the glomerulosa electrolyte-regulating mechanism has re-adapted to this new level of salt intake. That about 30% of captured wild rats drink 3 to 4 times as much water per day as do domesticated rats of the same weight (14) may be a result of increased activity in the wild rat glomerulosa layer.

The fasciculata and reticularis zones in the rat are known to be principally affected by ACTH stimulation or stress. They increase in width and lipid content following chronic mild stress (15). The hypertrophy and increased lipid content and aldehyde and carbonyl groups in these zones in the wild rat probably represents an adaptation resulting from the frequent stresses that confront the wild animal in its free environment. The hyperemia of the wild rat cortex may also reflect increased secretory activity. Brown-Séquard first reported congestion of the adrenal after trauma (16). Careful studies since then have shown that adrenal hyperemia may follow different forms of stress (17, 18). Recent experiments indicate that ACTH causes increased adrenal blood flow (19, 20, 21); however, Hartman, *et al.*, showed that vasopressin contamination of the ACTH preparations may be responsible for increases in adrenal blood flow they observed (21).

The wild rats in this experiment came from an unusually protected farm environment. However, there were stressing factors constantly present including its important natural enemies—the owls and dogs. City life with the presence of human enemies doubtless presents additional hazards to the rat which can account for the histological differences seen in the city rat.

The intracellular colloid which was present in the cortex of the farm rat

may represent an accumulation of some precursor or metabolic by-product of the cortical cells under conditions of excessive ACTH stimulation. Selye and Stone have reported its appearance in the cortex of the domesticated rats after administration of anterior pituitary extract alone or together with methyl-testosterone or desoxycorticosterone. These authors reviewed the literature and advanced the view that the colloid accumulations represent a storage phase (22). Rich has produced colloid accumulations in the domesticated rat cortex by administration of large doses of ACTH and cites its occurrence in a number of human autopsy cases (23). The colloid itself is not a pigment but a hyaline, amorphous protein accumulation which is eosinophilic and fuchsinophilic and somewhat resembles in its staining properties thyroid colloid (22). Selye and Stone suggest that colloid appears if the stimulus for hormone production is greater than that for granule discharge. This interpretation is compatible with our observations that the wild farm rats used for this study underwent less stress in their environment than did the city wild rat. The city rats which had no colloid storage probably had a greater demand for adrenal output. These interpretations are necessarily tentative until more is known about colloid in the adrenal cortex.

Capitivity presents a severe psychologic stress after the chronic stresses in the wild rat's free environment have already played their role in causing hypertrophy and hyperactivity. Nichols has shown that wild rats have a decrease in adrenal cholesterol content within hours after capture and a subsequent rise to above average levels after 24 hours in captivity (24). This secondary rise in cholesterol correlates with the histological appearance we have seen in the glands after several days or weeks of captivity. The cortical lipids increase and eventually fill in the transition zone and inner fasciculata and reticularis zones producing a dense uniform concentration of lipid throughout the cortex.

The persistence of fetal or provisional zone in an occasional wild rat is in accord with observations of Howard, who has observed that the X zone of the wild mouse persists longer than in the domesticated mouse (25). The pattern of development and regression of the provisional zone in the domesticated rat has been studied by Mitchell (26), and Bachmann has recently reviewed the literature on the provisional zone (27). The physiologic significance of this provisional zone is still undetermined.

SUMMARY

A comparison of the adrenals of wild Norway rats trapped in the city or shot on a farm and the adrenals of domesticated rats from our colony showed that the adrenals not only are larger in the wild than in the domesticated Norway rat, but they have a different shape and color. They are usually pyramidal in shape and somewhat elongated, rather than spherical and are brown, rather than pink. In the wild Norway rat adrenal the

following histological contrasts to the histological appearance of the domesticated rat adrenal were noted: a) the capsule is narrower, denser and has less connective tissue cells. b) the glomerulosa zone has larger cells containing more lipid, aldehyde and ketonic carbonyl groups. c) the transition zone is practically absent. d) the fasciculata zone is wider and contains more lipid, aldehyde and ketonic carbonyl groups; the fasciculata of the rats shot on a farm often contained intracellular colloid. e) the cell cords of the reticularis zone are straight and line prominent vascular channels; the reticularis cells contain lipid, aldehyde and ketonic carbonyl groups in greater quantities than seen in the domesticated rat. f) the medulla is often markedly vacuolated.

Wild rats trapped in the city and kept in captivity usually showed great increases in the lipid, aldehyde and ketonic carbonyl groups in the inner part of the fasciculata zone and throughout the reticularis zone. There was no intracellular colloid in this group of rats.

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THE EFFECT OF DEHYDRATION, STARVATION, AND PITRESSIN INJECTIONS ON THYROID ACTIVITY IN THE RAT[1]

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ONE of the usual effects of severe stress in experimental animals is depression of the activity of the thyroid gland (1–6). In studies of the role that the hypothalamus plays in this response it has been demonstrated that section of the pituitary stalk prevents the usual thyroid inhibitory reaction to pain or restraint in the rabbit (7, 8). Pituitary stalk section will also prevent the usual discharge of adrenocorticotrophic hormone (ACTH) which follows "emotional stress" in the rabbit (7, 9) or rat (10). Taking into consideration the absence of secreto-motor nerves to the anterior pituitary gland (11) it has been postulated that both the thyroid inhibition and the increased adrenal cortical activity which are observed following "emotional" stress are brought about through the effects on the anterior pituitary gland of neurohumeral substances transmitted by way of the hypophysial portal vessels (8, 9, 12).

The identity of these suspected neurohumors is not known at present, but a number of observations point to the possibility that the anti-diuretic hormone (ADH) or a substance closely associated with it in the neurohypophysis may be responsible for the release of ACTH in certain stressful conditions (13–18).

The following experiments were done in order to ascertain whether or not the anti-diuretic hormone was also responsible for the inhibition of the thyroid gland that occurs in some kinds of stress. In these studies, dehydration was used to stimulate the endogenous release of ADH, and the effects of exogenous ADH were elicited by injection of solutions of Pitressin. In analyzing the effects of dehydration on thyroid activity it was necessary to study thyroid responses to total and partial food deprivation since thirsty animals were found to eat less food than normal animals and food restriction per se is known to influence thyroid activity.

METHODS

Male rats of the Sprague-Dawley strain, weighing between 234 and 350 gm. were kept in a controlled temperature room at 26.6 ± 1° C. Photo-periods were constant, and rela-

Received September 8, 1956.

[1] This work was supported in part by United States Public Health Service Grant B-834(c).

[2] Lowell M. Palmer Fellow.

tive humidity varied between 30 and 68 per cent. The animals were fed a standard rat diet[3] and in some experiments were given a low iodine diet[4] with iodine supplements administered daily by subcutaneous injection of potassium iodide. In experiments on water deprivation, the drinking tubes were removed from the cages, and the animals allowed to eat the low iodine diet *ad libitum*. In experiments on food deprivation, free access to water was allowed.

Thyroid activity was determined by the measurement of the rate of release of I^{131} from the thyroid gland using an external counting technique in unanaesthetized animals similar to that used in the rat by Wolff (19) and in the rabbit by Brown-Grant, von Euler, Harris and Reichlin (20). The radioactivity of the neck region was counted twice daily beginning 48 hours after the intraperitoneal injection of 20 or 40 μc. of I^{131}. The animals were counted for 15 seconds in each of 4 positions in relation to a heavily shielded gamma detector (either a bismuth-cathode G-M tube or a scintillation counter).[5] The lead shield used was 24 mm. thick and counting was done over an oval aperature 12 by 18 mm. Metal guides or a yoke were used to ensure consistent position. With the dosage of I^{131} used, repeated counts of the neck region at the same time usually varied by less than $\pm 2\%$. Correction for radioisotope decay was made by comparison with an I^{131} standard. No correction for background radioactivity was made because in preliminary studies it was found that at 48 hours the non-thyroid neck tissues contributed less than 5% of the total neck count. Serum precipitable iodine (SPI) was determined by the method of Barker, Humphrey and Soley (21).

Some difficulty was encountered in the intra-muscular administration of Pitressin Tannate[6] in oil in that large amounts of the oil alone, when injected into the gastrocnemius muscle led to a marked slowing in the rate of release of I^{131} from the thyroid gland, presumably through a non-specific stress effect (6). Pitressin was administered in one of two ways. In one experiment, Pitressin in 0.1% acetic acid and 0.5% phenol was diluted tenfold with physiological saline immediately before the subcutaneous administration of 0.1 ml. amounts. In other experiments, Pitressin Tannate in oil containing 5 U/ml was injected subcutaneously. Control animals received identical quantities of the appropriate vehicle. Thyrotrophic hormone[7] was dissolved in physiological saline just before use, and was injected subcutaneously in a 0.5 ml. volume. At the termination of most experiments the thyroid and adrenal glands were dissected free, trimmed and weighed to the nearest 0.1 mg. on a torsion balance.

RESULTS

1. *The effect of dehydration on thyroidal I^{131} release*

The radioiodine release curves of 5 rats were determined in the usual way for a 3-day period. On the morning of the fourth day the drinking tubes were removed and the rats allowed access to their regular feed. Starting on the second day of dehydration (Fig. 1), a progressive decrease in slope

[3] Purina Laboratory Chow which contains, according to the manufacturer, at least 2 ppm of iodine, 23% protein, and 10–12% moisture.

[4] Low iodine test diet, Nutritional Biochemicals. Contains wheat gluten, 18%, dried pig liver, 2%, yellow corn meal, 78%, calcium carbonate, 1%, sodium chloride, 1%.

[5] Nuclear—Chicago, Illinois.

[6] Obtained through the kindness of Dr. D. A. McGinty, Parke, Davis Co., Detroit, Michigan.

[7] Lot #317-51 obtained through the kindness of Dr. J. R. Steelman, Armour Laboratories, Chicago, Illinois.

ensued, and by the end of the fourth day of dehydration the slope of the curve was very nearly zero. At this time the thirsty animals showed marked cachexia and undoubtedly were suffering from the circulatory effects of severe hemoconcentration. It was of interest therefore to determine whether the slowing of the rate of iodine release was due to a lack of responsiveness of the thyroid gland to thyrotrophic hormone (TSH) or to a decrease in TSH output. A partial answer was obtained by observing the thyroid response to exogenous TSH, 2 mg. of which were injected subcu-

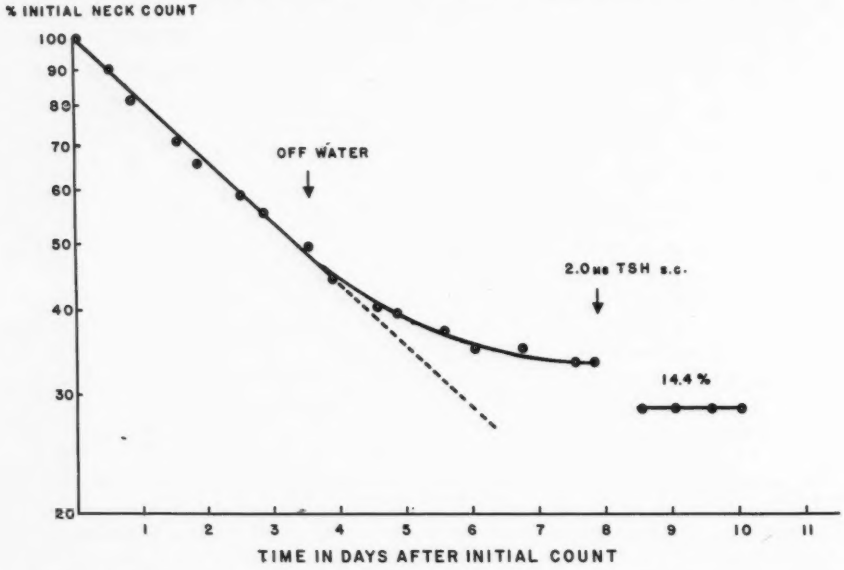

FIG. 1. The effect of complete restriction of water on thyroid I^{131} release in a group of five rats. The effect of exogenous TSH administration upon the slow iodine release rate in thirsted animals is also demonstrated. A reduction in thyroid I^{131} content of 14.4% was produced.

taneously in 0.5 ml. of 0.9% NaCl solution. Over the next 18 hours a discharge of I^{131} occurred which indicated that the thyroid gland was at least partially responsive to TSH. The average change in gland I^{131} content over this interval was 14.4%. For the next 2 days there was no further decrease in gland radioiodine content.

II. *The effect of dehydration on thyroidal I^{131} release in rats given large parenteral iodide supplements*

During the course of the first experiment urinary output fell, as did food intake. It was possible therefore, that the observed decrease in I^{131} discharge rate in Exp. 1 could be attributed to alterations in extra-thyroidal iodine metabolism rather than to any change in thyroid activity. This might be due to decrease in renal excretion of I^{131} from degraded labelled

thyroid hormone, and to increase in recirculation of I^{131} due to the low serum I^{127} in the semi-starved rats (22).

For these reasons, the effects of dehydration were studied in rats given large doses of I^{127} as potassium iodide (1500 mcg./day) s.c. in 2 divided doses. In this way iodine intake would be kept independent of food intake

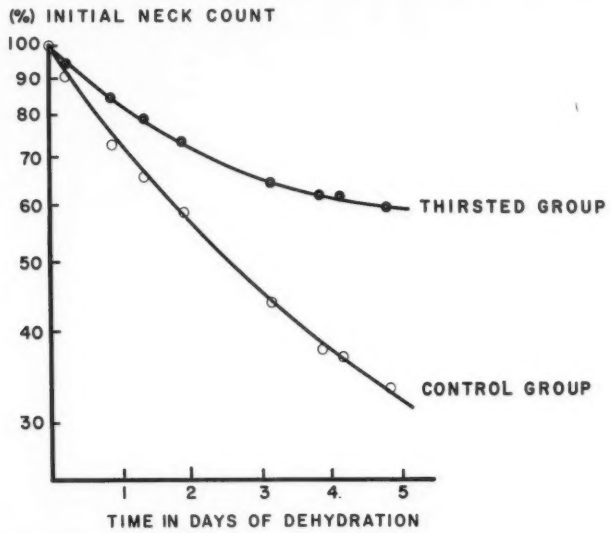

FIG. 2. The effect of complete restriction of water on thyroid I^{131} release in a group of 6 rats as compared with a group of 6 normal controls. Iodine intake supplemented by twice daily subcutaneous injection of 750 micrograms of KI. Thirst period began immediately after the initial neck count.

and the relatively large concentration of serum I^{127} by dilution would reduce the re-circulation of I^{131} from degraded labelled thyroid hormone (23). In this experiment the rats were divided into groups of 6 control and 6 thirsted animals, the period of dehydration commencing immediately after the initial neck count which was 48 hours after radioiodine injection. Both groups received the potassium iodide solution from the time of the first count.

Dehydration with constant high iodine intake induced a marked decrease in the rate of thyroidal iodine discharge (Fig. 2). Statistical analysis of successive neck counts indicates that by 33 hours the release rates of the two groups of rats have become different at the .01 level of significance.

III. *The effect of dehydration on thyroidal I^{131} release as compared with pair-fed controls*

During the course of experiments on dehydration there was a gradual falling off of food intake. It is well known that starvation is associated with a decrease in thyroid activity (24, 25) and it was important to determine

the extent to which the voluntary food restriction of the thirsted rats contributed to the observed decrease in thyroid activity. This was done by comparing I^{131} release curves in groups of 8 thirsted rats allowed low iodine diet *ad lib.*, 8 pair fed controls allowed tap water *ad lib.* and 6 control rats allowed low iodine diet and tap water *ad lib.*

Animals were given the low iodine diet so that a constant daily iodine intake could be assured, regardless of food intake, by the parenteral administration of 30 mcg. of potassium iodide in 0.2 ml. of physiological saline. Both thirsted and semistarved animals showed a reduction in thy-

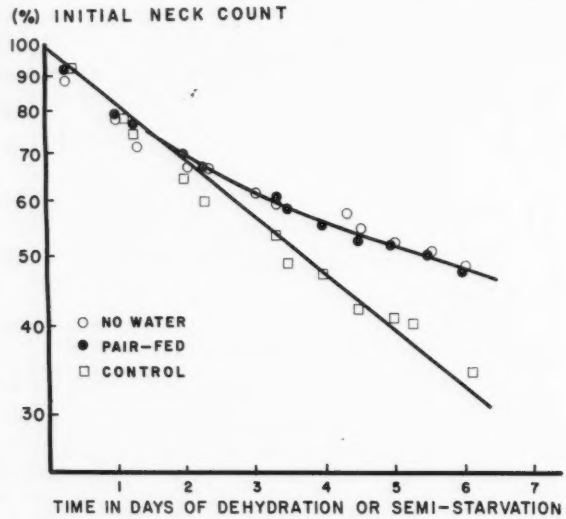

FIG. 3. The effects of water deprivation on thyroid I^{131} release as compared with pair fed and *ad lib.* fed control animals. There are 8 thirsted, 8 pair fed animals, and 6 control animals. Low iodine diet offered, constant iodine intake in all groups maintained by once daily injection of 30 mcg. of potassium iodide.

roid activity as compared with control animals (Fig. 3), but there was no difference between the rates of release in pair fed (semi-starved) rats allowed free access to water, and in thirsted rats with self imposed food restriction. The pair fed animals drank more water than did the control animals for the first two days, and approximately the same quantities of fluid for the next two days (Fig. 4). It is only on the last 2 days that water intake fell below normal values. Thirsted animals showed progressive decrease in food intake, which fell to half on the second day, and was almost zero beyond the fourth day. The control animals gained weight during the experimental period. During the first day the thirsted animals lost more weight than did the pair fed control animals, but thereafter the rate of weight loss was the same. Judging from general vigor, condition of coat and

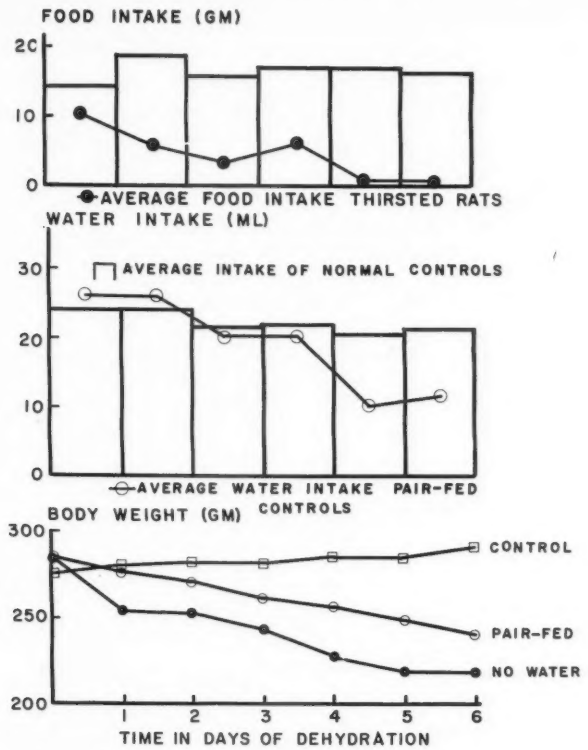

FIG. 4. This figure demonstrates the food consumption by days of a group of 8 rats deprived of water, the water consumption of 8 rats pair fed with the thirsted animals and the food and water consumption of a group of 6 normal rats. Body weight changes are also illustrated. The bars represent the average control value.

behavior the thirsted animals appeared far sicker than did the pair fed controls.

IV. *The effects of dehydration on thyroidal I^{131} release as compared with complete starvation*

In the foregoing experiment, dehydration plus semi-starvation had no greater effect on thyroid activity than did an equivalent degree of semi-starvation alone. The relation between the effects of starvation and of dehydration on thyroid activity were studied further by comparing iodine release rates in completely starved, in thirsted and in control animals at two different environmental temperatures (Figs. 5, 6). As in Experiment III, a low iodine diet was offered the control and thirsted animals, and all 3 groups received 30 mcg. of potassium iodide per day by subcutaneous injection. There were 7 or 8 rats in each group. In the experiment carried out at $26.6 \pm 1°$ C (Fig. 5), starvation alone led to a more prompt effect on io-

dine release than did dehydration, this lag period approximately corresponding to the interval in which the thirsty animals consumed more than half of the usual amounts of food. At the 36th hour and thereafter, the differences between all 3 groups are statistically significant at the 0.01 level

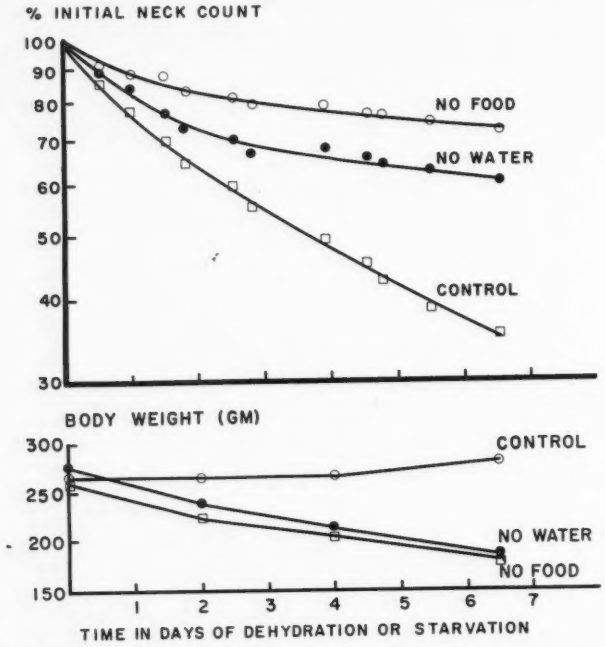

FIG. 5. The effects of water deprivation and of starvation on thyroid I^{131} release and on body weight as compared with normal animals. Room temperature $26.6 \pm 1°$ C. There are seven or eight animals in each group. Low iodine diet offered to thirsted and control groups. Constant iodine intake in all groups was provided by twice daily injection of 15 mcg. of potassium iodide. Experimental treatment began immediately after the initial neck count.

or greater. After the third day the slopes of the curves of starved, and thirsted rats are parallel.

At the end of the experiment the animals were decapitated, and serum precipitable iodine (SPI) was measured from pooled sera of the three groups. The SPI levels observed corresponded with the findings of the release curves in that the fasted group had the lowest SPI (2.4 mcg./100 ml.), in contrast to a SPI level of 3.1 mcg./100 ml. in the thirsted animals, and of 4.4 mcg./100 ml. in the normal animals. These values are the average of two determinations for each pool, but because the range of individual values within each group is not known, it is not possible to evaluate the significance of these differences.

This experiment was repeated in an identical fashion except that the

animals were counted only once a day and were kept at a temperature of $21 \pm 1°$ C instead of the usual $26.6 \pm 1°$ C. There was an increased rate of thyroid activity as compared with animals housed at 26.6° C (Fig. 6), but with this exception the same relations were apparent. The starved group that was allowed free access to water showed the earliest changes in thy-

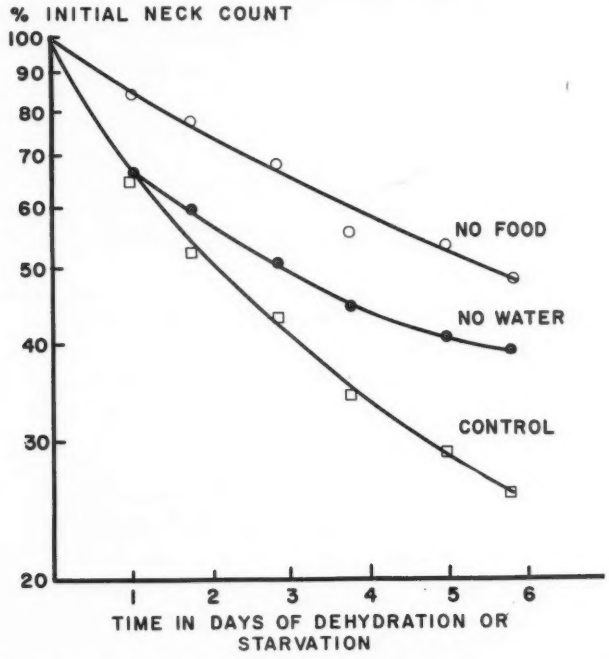

FIG. 6. The effects of water deprivation and of starvation on thyroid I^{131} release as compared with normal animals. Room temperature $21 \pm 1°$ C. There are eight animals in each group. Low iodine diet offered to thirsted and control group. Constant iodine intake in all groups was provided by twice daily injection of 15 mcg of potassium iodide. Experim ntal treatment began immediately after the initial neck count.

roid activity, while the thirsted animals again showed a lag period of at least 24 hours before displaying a slope equal to that of starved animals. The totally starved animals lost weight at the same rate as did the thirsted animals (Fig. 7), and drank progressively less water from day to day. The thirsty animals showed a progressive decline in food intake beginning with the first day of the experimental period.

V. *The effect of hypertonic saline ingestion on thyroidal I^{131} release*

In the foregoing experiments it was demonstrated that decreased food intake was the chief determinant of the level of thyroid activity in experimental dehydration without regard to state of hydration. In an attempt to

elicit an increased antidiuretic hormone release with reasonably well maintained nutrition the effect of hypertonic saline ingestion was studied. From histological findings in the rat, Chambers (26) concluded that the drinking of hypertonic saline (1.5 to 2.5% NaCl) led to a discharge of antidiuretic hormone and other evidences of posterior pituitary activity, a conclusion

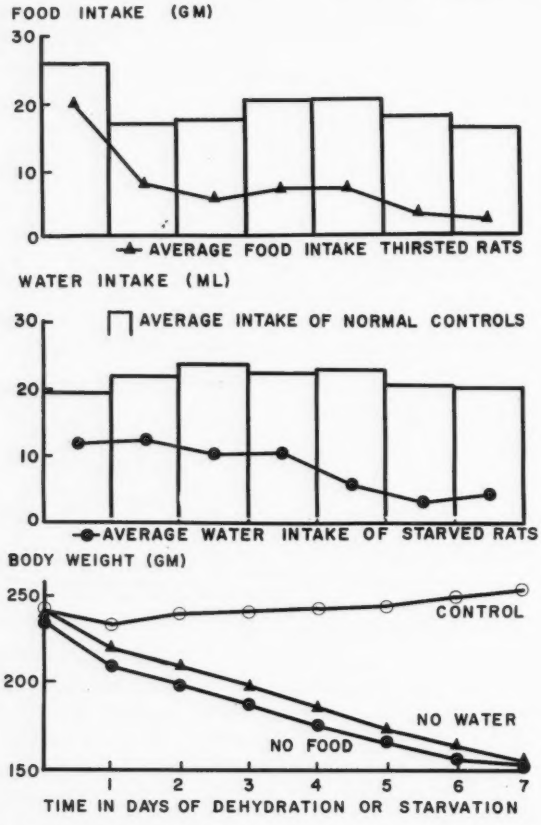

FIG. 7. This figure illustrates the effect of water deprivation on food intake and the effect of food deprivation on water intake as compared with normal animals. Body weight changes in the three groups of animals are shown. Animal room temperature $21 \pm 1°$ C.

previously reached by Selye and Hall (27) and Swan and Penner (28). It is also known from Adolph's studies (29) that rats can survive for prolonged periods on hypertonic saline solutions of 0.37 *M* and below, this being accomplished by the secretion of a maximally concentrated urine. In preliminary studies, it was found, in agreement with Adolph, that rats consumed little or no 3% saline. These animals showed I^{131} release curves which were identical with those of rats offered no fluid at all to drink. On

the other hand 2% saline (.34 *M*) was tolerated by most animals, and was used in this experiment.

I^{131} release curves were determined in 7 rats given 2% NaCl in their drinking water and in 7 control animals offered tap water *ad lib.* Purina diet was offered *ad lib.* and no parenteral iodine supplements were ad-

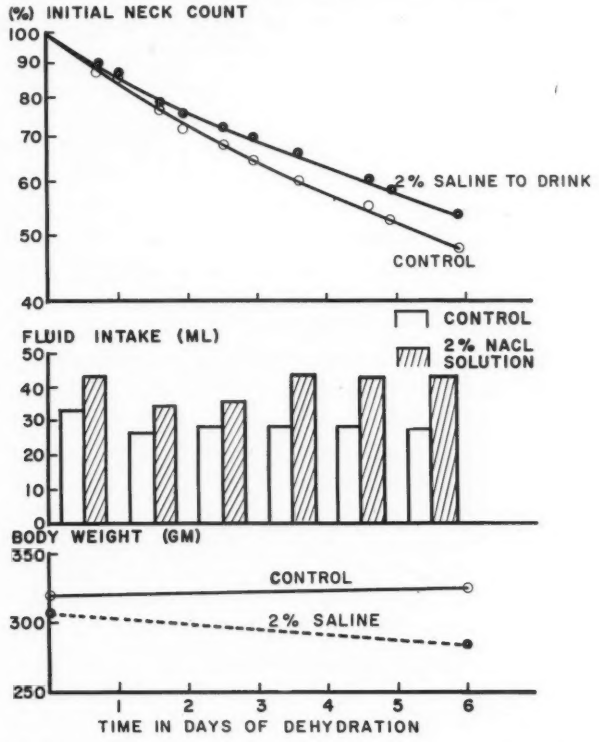

FIG. 8. This figure illustrates the effect of hypertonic saline ingestion on thyroidal I^{131} release, daily fluid consumption and body weight as compared with control animals. There are 6 control rats and 5 rats receiving 2% saline instead of tap water immediately after the initial neck count. Differences between individual points at each time interval are not significant statistically.

ministered. Two of the seven rats offered salt water drank considerably less than the average control intake (12.3 and 8.5 ml./day vs. 28.6 ml./day) and were not included in this series. The other animals all drank more than the average of control rats (40.5 vs. 28.6 ml./day). The rats offered salty drinking water lost weight during the experimental period but this weight loss (6.5%) was considerably less than that of a comparable thirst experiment (Experiment II, 33.9%), indicating some measure of physiological adjustment to the demand for water conservation. The release

curves (Fig. 8) show only a small difference between rats drinking salt water and control rats which is not statistically significant. Food intake was not measured in this experiment, so that it is not possible to know to what extent voluntary food restriction was responsible for the weight loss, and slight decrease in thyroid activity that were observed.

VI. *The effect of Pitressin injections upon thyroidal I^{131} release*

The major question to be answered by this investigation was whether or not the amounts of antidiuretic hormone released in extreme dehydration exerted an effect upon thyroid activity. In this group of experiments, amounts of Pitressin containing quantities of ADH calculated to equal or exceed the output of the posterior pituitary gland in extreme dehydration were injected and the effect on I^{131} release observed. The theoretical considerations involved in choosing the doses used are mentioned below in the discussion. The total dose that could be given was limited by the fact that large doses of oily vehicle injected into the gastrocnemius were found to result in marked slowing of thyroid I^{131} release. Three series of experiments were carried out to study the effects of Pitressin on thyroid activity. In the first experiment 4 control rats were compared with 4 rats given 0.5 mU. and 4 rats given 100 mU. Pitressin Tannate in oil twice daily by subcutaneous injection. In the second experiment 7 control rats were compared with 7 animals given 156 mU. and 7 animals given 312 mU. of Pitressin in watery solution twice daily by subcutaneous injections. In the third experiment 9 control rats were compared with 9 rats injected subcutaneously with Pitressin Tannate in oil at 24 hour intervals. A dose of 1000 mU. in 0.2 ml. was given for 3 days, followed by 2000 mU. for the next 4 days. In none of these experiments was there a demonstrable difference in I^{131} release rates between Pitressin injected and control animals.

VII. *The effect of Pitressin injections upon thyroidal I^{131} uptake*

Pitressin in amounts up to 2000 mU. per day did not influence the release of I^{131} from the thyroid gland. It was of interest to determine whether or not ADH release was of importance in producing the acute inhibition of thyroid iodine uptake seen within one hour of some kinds of stress both in normal rats (4) and in hypophysectomized rats (30). The effect on the thyroid radioiodine uptake of large doses of Pitressin was therefore studied. In this experiment a group of 9 rats were injected subcutaneously with 5 U. of Pitressin in normal saline solution. Ten minutes later, 5 μc. of I^{131} were administered intraperitoneally, and following an interval of one hour the animals were killed, the thyroid glands excised, and the radioiodine content measured in a well scintillation counter. Uptake measurements were compared to a group of 8 control animals. Within ten minutes of the injection of 5 U. of Pitressin marked respiratory difficulty and striking pallor of the nose and extremities were noted, these changes lasting for

from 30 minutes to one hour. Despite the evidence that Pitressin was acting at a "pharmacological" level, the I^{131} uptake in Pitressin injected animals was $6.01 \pm .96\%$ as compared with $6.86 \pm .38\%$ for the control animals, values which are not significantly different. Serum radioiodine concentration 70 minutes after Pitressin injection was $0.039 \pm .012$ μc./ml. as compared with $.070 \pm .003$ μc./ml. in the control group, thus indicating that the Pitressin, although exerting some effect on extra-thyroidal iodine metabolism had not materially influenced the percentage uptake by the thyroid gland.

VIII. *The effect of dehydration, starvation, and Pitressin injections on thyroid and adrenal gland weight*

In general, it can be said (see Table 1) that dehydration leads to a marked decrease in thyroid weight (Experiments I, II, III, IV), and to increase in adrenal weight as compared with that of control animals. These differences are statistically significant. As shown by calculation of gland weight body weight ratios, both absolute and relative adrenal hypertrophy is noted, while the thyroid gland though decreasing in size does not show as large a proportional loss as does body weight. In comparing the effects of

TABLE 1. BODY WEIGHT, THYROID WEIGHT AND ADRENAL WEIGHT CHANGES FOLLOWING DEHYDRATION, STARVATION AND PITRESSIN INJECTIONS

Experiment	No. of animals	Body weight, gm.		Thyroid weight			Adrenal weight		
		Initial	Final	Mg.	S.E.	Mg./10 gm. b.w.	Mg.	S.E.	Mg./10 gm. b.w.
I. Control[1]	10	276	287	18.6	0.97	0.65	36.9	1.56	1.29
Thirsted+TSH	5	255	180	12.8**	0.30	0.71	43.6**	4.6	2.42**
No iodine Supplement									
II. Control	6	256	282	20.3	2.04	0.72	35.2	1.65	1.25
Thirsted	6	253	167	13.3**	0.55	0.87	40.9**	1.19	2.45**
KI 30 mcg./day									
III. Control	6	275	291	24.5	0.89	0.84	36.5	4.56	1.25
Thirsted	8	285	209	17.5**	0.69	0.87	51.0**	3.03	2.44**‡
Pair-fed	8	282	240	17.5**	2.38	0.74	45.4	1.97	1.88**‡
KI 30 mcg./day									
IV. Control	8	265	271	22.5	1.02	0.84	32.6	1.02	1.23
Thirsted	7	273	186	16.7*	1.79	0.88	42.5**†	0.07	2.25**‡
Starved	8	258	180	18.3**	0.92	1.03†	35.6**†	1.35	1.99**‡
KI 30 mcg./day									
V. Control[2]	6	317	327	21.2	0.99	0.65	40.8	1.98	1.27
2% NaCl[3] to drink	5	306	286	18.1*	0.96	0.63	39.0	2.16	1.39
VI. Pitressin in Saline									
Control	7	279	289	21.3	1.30	0.73	35.3	1.37	1.22
0.312 u./day	7	275	289	20.5	0.80	0.71	38.9	2.13	1.34
0.624 u./day	7	274	289	21.3	0.91	0.73	37.8	1.89	1.31
Pitressin Tannate in Oil									
Control	9	322	334	23.4	1.29	0.70	38.1	1.65	1.14
Pitressin[4]	9	317	318	21.0	1.32	0.66	36.8	1.38	1.16

[1] Similarly counted control animals.
[2] Seven rats in group but organ weights available for six animals.
[3] Including only those animals drinking amounts of fluid equal to or greater than control animals.
[4] Pitressin Tannate in Oil. 1 u. daily for 3 days, followed by 2 u. daily for 4 days.
* Significance of difference as compared with control group $P<0.05$.
** Significance of difference as compared with control group $P<0.01$.
† Significance of difference between starved and thirsted group $P<0.05$.
‡ Significance of difference between starved and thirsted group $P<0.01$.

starvation with those of dehydration Experiments III and IV show that thirsted animals have larger adrenals than rats starved for a comparable period of time. In Experiment III there is no difference in the thyroid weight between thirsted and pair fed animals. In Experiment IV, rats starved for 7 days have thyroids which are 8.7% larger than animals thirsted for a similar period, but this small difference is not statistically significant.

The ingestion of 2% sodium chloride for 6 days led to a 14.7% decrease in thyroid gland size which was not statistically significant ($P > 0.05$). There was no effect on adrenal gland size.

The administration of Pitressin in 3 dosage schedules (Exp. VI) failed to produce a significant effect on either thyroid or adrenal weight.

DISCUSSION

Radioiodine release curves have been utilized in these experiments as the principle measure of thyroid activity. The usefulness of this method has been established by the studies of Wolff (19), Perry (23), and Albert (31) in the rat and those of Brown-Grant *et al.* (6, 20) and Reichlin and Reid (32) in the rabbit. Under conditions of dehydration or starvation, I^{131} release rates must be interpreted with caution because of the known dependence of the release curve upon iodine content of diet (33) and upon recirculation of I^{131} from degraded thyroid hormone. Either a decrease in iodine content of the diet, or increase in percentage recirculation of I^{131} due to changes in extra-thyroid iodine metabolism might conceivably result in a decrease in slope of the release curve. These non-thyroid factors were probably not responsible for the slowing of iodine release that was observed in these experiments however, because in all but the first experiment, adequate parenteral iodine supplements were administered and in one experiment, a dose of I^{127} was used which would, by dilution tend to reduce the recirculation of I^{131} from degraded hormone. The changes in release curves moreover were confirmed by the parallel changes in thyroid weight and in S.P.I. levels, and are in agreement with the findings of Meites and Wolterink (34) who demonstrated that 7 days of starvation led to decreased I^{131} uptake.

These experiments were carried out primarily to determine the effect of anti-diuretic hormone (ADH) upon thyroid activity. Anti-diuretic hormone secretion is increased markedly by the complete restriction of water as studied in these experiments (35–38). Under conditions of dehydration, both I^{131} release rates and thyroid size were observed to be decreased. This could not be attributed specifically to the effects of ADH however, because the changes in thyroid function which were observed were no different from those seen in normally hydrated rats with food restriction of a degree comparable to the low voluntary food intake of thirsted animals (Exp. III). As judged by general appearance, severe hemoconcentration and adrenal hypertrophy, total fluid restriction constituted a far more

profound stress than did partial starvation in pair fed controls with free access to water. The failure of dehydration to produce a greater reduction of thyroid activity than does semi-starvation is not due to an already maximal thyroid inhibition since thyroid I^{131} release rates are reduced by hypophysectomy or by thyroxine administration to less than 2.7%/day (19). In this laboratory the I^{131} release rate of thyroxine treated rats is less than 1%/day (unpublished observations).

The role played by starvation in bringing about the effects observed following severe dehydration is further shown in Exp. IV in which reduction in thyroid activity began at least a day later in thirsted animals than in starved animals with comparable degrees of weight loss, this time lag corresponding to the period over which rats offered no water will consume more than half of the normal amount of food. After one or two days, (depending on the environmental temperature), the iodine release rates in thirsted rats became equal to that of starved animals. The findings suggest that food intake and not the degree of dehydration is the major determinant of thyroid activity under these conditions.

It is of interest that restriction of any one of the three major metabolic needs, water or food as in these experiments, or oxygen (39) results in inhibition of the thyroid gland, an adjustment of obvious adaptive value. In fact, low oxygen supply may exert its thyroid depressing effects also through changes in food intake since in the studies of Gordon *et al.* (39) the animals lost weight when exposed to low oxygen tensions, and Van Liere (40) points out that at high altitudes appetite is very poor, and that beyond a critical altitude animals will not eat. The importance of dietary changes in influencing experimental studies of thyroid activity has been recently demonstrated by Clifton and Meyer (41).

In experiments in which rats were offered hypertonic saline instead of tap water to drink, larger than normal volumes of drinking fluid were consumed which indicates, presumably, that the amounts of 2% saline drunk were sufficient to furnish the usual available water, and in addition to carry away in urine the extra salts ingested (29). That this homeostatic response involves increased ADH release is probable (26, 28, 42). Since the animals lost weight during the period of saline ingestion, it is likely that food reduction was of importance in this experiment as in the preceding ones in causing the slight differences observed in thyroid activity. Unfortunately, food intake was not measured in this experiment, and pair-fed controls were not utilized, so that no hard and fast conclusions can be drawn from these observations.

It is possible that the concomitant starvation, or inanition prevented any specific ADH effects on TSH release during induced dehydration. This explanation is not likely however because the effects of dehydration follow so exactly in time sequence the effects of the associated starvation, and Pitressin injections containing ADH in amounts up to 2 U. per day exerted no effect on thyroid activity.

This amount of Pitressin is thought to exceed by a considerable margin the quantities secreted under conditions of extreme dehydration, which have been estimated from the reports of Noble, Plunkett and Taylor (37) (erratum in printing corrected by personal communication, Noble, 1955), and that of Dicker (43). According to Noble a very approximate figure for the amount of ADH secreted in the third 24-hour period of dehydration is 720 micro U./Kg./min. which is 1.03 U./Kg./24 hr. Since the animals weighed 250–300 gm. the dosage of 2 U./day (provided it was all absorbed) was 6 to 8 times the figure suggested by Noble.

In Dicker's experiments the infusion of 200 micro U./Kg./min. led to a maximum antidiuresis. This figure is approximately two-sevenths that of Noble, and corresponds to an amount equal to 0.086 U./24 hr. for a 300 gm. rat. Following intra-muscular injection, Wilson and McGinty (44) found that 0.180 U. of Pitressin Tannate in oil induced an effective antidiuretic action 16 hours after administration. It should be stated however that there is an unavoidable uncertainty as to the time course and percentage absorption of Pitressin and Pitressin Tannate from the subcutaneous depot.

Amounts of Pitressin which produced intense peripheral vasoconstriction and respiratory difficulty were found to exert no significant effect on the one hour uptake of I^{131} by the thyroid gland. This is interpreted as indicating that the acute depression of thyroid function which has been reported to follow tourniquet shock in normal rats (4), or to follow forced muscular exercise in hypophysectomized rats (30) is not due to any local vascular or metabolic effect of ADH acting directly on the thyroid gland.

These experiments suggest that amounts of ADH released under conditions of extreme dehydration exert no specific effect on thyroid activity. It is not absolutely certain however that these findings completely exclude ADH as a factor affecting the release of TSH from the pituitary gland as it is possible though not likely that severe dehydration may not represent the maximal stimulus to ADH production. Mention should be made of the possibility that local concentration of ADH in the virtually undiluted portal venous blood may be considerably higher than that produced by Pitressin injection, or the endogenous release of ADH from the pars nervosa. That the antidiuretic hormone is probably not involved in the regulation of thyroid activity is also indicated by the fact that hypothalamic lesions in rats may abolish compensatory thyroid hypertrophy without producing diabetes insipidus and diabetes insipidus may occur without interferring with compensatory thyroid hypertrophy (45).

In considering the effect of starvation upon thyroid activity it is of considerable interest that slowing of radioiodine release began as early as 24 hours in experiments at both 21 and 26.6° C, the difference between neck counts at this time and later being highly significant ($P < .01$). This early effect on iodine release confirms D'Angelo's observation (46) that acute

starvation in rats leads to marked decrease in TSH blood levels within 3 days. By the third day at 26.6° C and by the second day at 21° C the rate of iodine release reached a constant level of 2.5% and 11.9% per day respectively. The iodine discharge rate in starvation can be compared with that seen following total hypophysectomy, 2.7%/day, (19), 1.06%/day, (47), or thyroxine administration (less than 2.7% per day (19) less than 1%/day in this laboratory). This comparison would indicate that starvation does not abolish TSH release entirely, particularly as the environmental temperature is lowered. Although thyroid activity is reduced in severe starvation, the maintenance of body temperature seems to have first priority in determining the level of thyroid activity.

SUMMARY

In an investigation of the possible importance of the antidiuretic hormone in the regulation of pituitary thyrotrophic activity the effect on thyroid function of dehydration and of Pitressin injections has been studied. Water deprivation led to progressive decrease in thyroidal I^{131} release rate, to thyroid gland atrophy and to lowered serum precipitable iodine. The inactive thyroid gland of severely dehydrated rats was still capable of responding to exogenous thyrotrophic hormone. The changes in thyroid release rate and thyroid weight seen in dehydrated rats were identical to those in pair fed (and hence semi-starved) controls which were not dehydrated. In contrast with the more prompt thyroid inhibition seen in completely starved rats, there is a further delay of about 24 hours before I^{131} release rates in dehydrated rats fell to those of starved animals. During the first day of dehydration rats eat at least half the normal food intake, but food consumption falls off rapidly thereafter. Injections of Pitressin in amounts up to 2 U. per day exerted no effect on thyroid iodine release. Furthermore, the injection of 5 U. of Pitressin which produces intense peripheral vasoconstriction, and respiratory difficulty exerted no significant effect on the one hour thyroid uptake of I^{131}. The conclusion was drawn from these findings that the major determinant of thyroid activity under the conditions of the experiment was the level of food intake without regard to degree of hydration or dehydration. In conjunction with the failure of Pitressin in large doses to influence the thyroid gland these findings suggest that antidiuretic hormone exerts no specific effect on thyroid activity.

Starvation led to a prompt decrease in thyroid activity within 24 hours at 21° C and within 36 hours at 26.6° C. Iodine release rates are more rapid at lower environmental temperatures, even in starved animals. Adrenal weight changes indicate that severe dehydration brings about a greater release of ACTH than does starvation, while the changes in thyroid function appear to be related to level of food intake rather than water deprivation.

Note Added in Proof

While this article was in proof, A. Arimura, Y. Takagi and T. Ueno (Japanese J. Phys., *6*, 284, 1956) reported that in the rat, injections of vasopressin in doses of 80 mU/100 gm. body weight per day led to depression of the PBI with little or no change in metabolic rate. It is not possible at present to reconcile these findings with the demonstrated lack of effect on thyroid I^{131} release of Pitressin in doses up to 2000 mU per day in the present experiments.

Acknowledgment

The technical assistance of Mrs. Christa Kinderman is gratefully acknowledged.

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CONTROL OF CORTICOTROPHIN RELEASE: FURTHER STUDIES WITH *IN VITRO* METHODS[1]

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THE existence of a neuro-humoral mediator of hypothalamic origin, involved in the activation of the anterior lobe of the pituitary for the release of corticotrophin (ACTH) has long been postulated. This has been based on the anatomical study of the vascular and nervous connections between hypothalamus and adenohypophysis (1, 2, 3, 4) and on a highly suggestive series of experiments where pituitary-adrenocortical function was assessed after electric stimulation (5, 6, 7), stereotaxic destruction of various regions of the hypothalamus (5, 6, 7, 8, 9), or after severance of the normal connections between diencephalon and pituitary (3, 5, 10). The recent demonstration of ACTH-releasing activity in blood originating primarily from the hypothalamo-pituitary portal vessels (11) and the results of *in vitro* techniques using tissue cultures (12), organ cultures (13, 14) or short term incubation (15) of the adenohypophysis with living tissues (12, 13, 15) or homogenates (13) of the hypothalamus have contributed additional data in accordance with this concept.

However, an appreciable number of reports (6, 9, 10, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25) have called attention to the fact that the hypothalamic control of the pituitary for ACTH-release during stress may not be absolute and exclusive; intense surgical stresses appear to be still effective in eliciting a discharge of corticotrophin after extensive lesions of the whole ventral hypothalamus, of the median eminence or after transplantation of the adenohypophysis in the anterior chamber of the eye. No effort to reconcile these results with those showing involvement of the hypothalamus seems to have been made, with the single exception of Fortier's theory for a multiple control of the pituitary in which the nature of the stressor is in-

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Received September 12, 1956.

[1] Supported in part by funds provided under Contract AF 41(657)-17 with the USAF School of Aviation Medicine, Randolph Field, Texas, by the Blue Bird Circle Children's Clinic, Houston, Texas, and by a grant from the Lilly Research Laboratories, Indianapolis, Indiana.

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volved, neurogenic (emotional) stressors being mediated through the hypothalamus in stimulating ACTH-release, whereas others (systemic, nonspecific stressors) act through another pathway (10). On the basis of their *in vitro* studies (15, 26) and of Rothballer's morphological observations (27), Saffran, Schally and Benfey suggested that the posterior lobe of the pituitary plays an important role in the stimulation by stress of the anterior lobe of the pituitary for ACTH-release. They hypothesized, however, (26) that the posterior pituitary is only a relay between the hypothalamic nuclei and the pars distalis for storage and excretion of an ACTH-releasing factor (CRF) as it appears to be in the case of vasopressin and oxytocin.

This report deals with the isolation from the hypothalamus of a purified material which releases corticotrophin *in vitro*. That the ACTH-releasing activity as observed in this system may not have an exclusive hypothalamic origin will be evident from some of the control studies reported here and also from data with various preparations of substance P of von Euler and Gaddum (48).

MATERIAL AND METHODS

The rats utilized were females, weighing 90–175 gm., obtained from the Holtzman Rat Co., Madison, Wisconsin. They were kept in a constant temperature room for at least 48 hours before being utilized. At time of experiments, the rats were decapitated, the pituitary removed first (45–60 seconds), adrenals afterwards (2 to 3 minutes). No nembutal was given.

1. *Stimulation of ACTH-release and assessment of ACTH-release, by in vitro pituitary. Statistical treatment of the assays*

With important changes in the design of the bioassay, the short term incubation technique of the pituitary for ACTH-release reported by Saffran and Schally (15) was utilized in these experiments. It was considered more elegant and easier to assess statistically than the *in vitro* method we originally proposed (13, 28).

The method consists in incubating in 1 ml. of Krebs-Ringer bicarbonate medium (in two 20 ml. pyrex beakers) the halves of the anterior lobes of 6 rat pituitaries. The substance to be tested for ACTH-releasing activity, usually as a solution in distilled demineralized water at a concentration of 1 mg./ml., is added to one of the flasks; the volume of the other is equalized with water except when the volume added is below 10 μl. Both flasks are placed in one of the two chambers of a Dubnoff incubator at 37° C, under a moist atmosphere of 95% O_2, 5% CO_2, for approximately 60 minutes. At the end of the incubation period, the fluids are collected separately in test tubes containing 30 μl. of 1 N HCl, and the pituitary fragments are weighed to the nearest 0.01 mg. After neutralization with 30 μl. of 1 N $NaHCO_3$, 50 μl. and 150 μl. of the pituitary incubation fluids are then distributed as S_1, U_1 and S_2, U_2 in the *in vitro* assay for ACTH as described by Saffran and Schally (29), in which estimation of the release of (ring A-Δ4, 3 keto) steroids by fragments of rat adrenal glands is taken as the criterion of ACTH activity. The volumes of 50 μl. and 150 μl., calculated for 33.3 mg. of adrenal tissue in the corresponding flasks are adjusted to the weight of the adrenal fragments in each flask (see 29). Incubation of the adrenal fragments is run in a Dubnoff shaking incubator (not in Warburg vessels). Calculations of the final results include adjustment for the discrepancies in volumes of the adrenal-containing flasks and discrepancy in the weight of the pituitary

fragments.[5] The bioassay for ACTH is a four point assay in duplicate and is calculated according to Bliss (30).

All data have been expressed in "potency" of the stimulated pituitary as related to the amount of corticotrophin released by the unstimulated pituitary to which was attributed a value of 1 or 100%. The results therefore, are not expressed in units of U.S.P. Reference Standard for Corticotrophin. In testing pituitary tissue against itself rather than against U.S.P. Reference Standard for Corticotrophin in each experiment, one eliminates possible variations in responsiveness of adrenals and pituitaries in the different batches of animals (such variations do exist as demonstrated by the variations in the slope *b* and the parameter *a* of the curves in different assays). This allows the expressed potency to have true confidence limits.

2. *Extraction and purification of the ACTH-hypophysiotropic fraction*

a. *Preparation of fresh tissues.* Hypothalamic tissue, brain cortex (frontal, temporal, occipital lobes) and cerebellum from pork and beef were obtained in local slaughter houses. The brains were received as soon as possible (10–15 min.) after the anima's were killed by exsanguination. A large piece including the whole hypothalamic region and optic chiasm ventrally, corpora quadrigemina and geniculate bodies dorsally was rapidly cut with scissors and immediately frozen in dry ice powder. The same procedure was utilized for fragments of brain cortex and cerebellum. The tissues were kept in dry ice from a few hours up to several weeks, without appreciably different results. Hypothalamic tissues obtained 45 minutes after the death of the cattle did not yield active material. The fragments containing the hypothalamus were thawed to a consistency permitting dissection and were rapidly trimmed of adjacent tissues. A block was cut out, limited by optic chiasm and substancia perforata anteriorly, hypothalamic fissures laterally and mammillary bodies posteriorly, and was opened along the midline to expose the third ventricle. Exposure of the walls of the third ventricle permitted easy identification of the anterior commissure, massa intermedia, posterior commissure, descending bundle of fornix, mammillary bodies, inner tuberal nuclei and median eminence. Magnifying glasses were necessary when dealing with hog hypothalamus. The fragments finally saved, included all the above mentioned landmarks with the exception of massa intermedia, the inferior limit of which thus constituted the upper line of the fragment.

Extraction procedures were started with a minimum of 50 gm. of hypothalamic or other brain tissues. Two methods of extraction were utilized:

1. The tissues were treated according to the method of Kamm *et al.* (31) originally described for the extraction of vasopressin and oxytocin from posterior pituitary tissue, the final product being extracted as in 2*c*.

2. A much shorter procedure was also utilized which consisted in extracting the *fresh tissues* in a Waring blender 5 times in 10 parts of 0.5% acetic acid, for 15 minutes at room temperature. The resulting extracts were pooled, frozen in heavy centrifuge bottle overnight, thawed the next morning, centrifuged at high speed for 30 minutes, after which the supernatant was lyophilized, defatted with petroleum ether or CCl_4 and processed as described in *2c*.

b. *Processed starting materials.* Commercial Pitressin (Parke, Davis lots #X-191404 and 191404), crude Protopituitrin (Parke, Davis lots #116384 and S1684L), acetone dried powders of hog and beef posterior pituitary, and two samples of substance P were also used as starting material. In a series of experiments, Protopituitrin and the acetone

[5] The correction factor introduces the log of the ratio of the weight of the pituitaries s/u, [log dose U_1, U_2+log K(s/u)], where K=1. The assumption has been made that the amount of ACTH released by two pituitary populations identical except for a difference in weight is proportional to the weight of tissue, within the limits of the small variations encountered.

dried powders were extracted as described in *2c*; the acetone dried powders of posterior pituitary were also treated by the Kamm procedure and the final product was extracted as in *2c*. Commercial Pitressin and the two samples of substance P were directly subjected to chromatography as described in *2d*.

c. *Final extraction of the active fraction.* Protopituitrin and the end-product of the Kamm procedure (or of its modification) were extracted three times at room temperature with 90% methanol. After centrifugation, the supernatant was poured off and the methanol evaporated *in vacuo* at 35–40° C in a water bath. This methanolic extract contains the material stimulating ACTH-release.

d. *Chromatography.* The methanolic extract was weighed, taken up in water and applied for chromatography on Whatman paper #1 or 3MM, at room temperature for 12–15 hours in an ascending system made up of: acetone 60 parts, diethylene glycol 10 parts, 0.5% aqueous urea 30 parts (solvent I). Side strips of each sheet were developed with ninhydrin in butanol and the desired fraction eluted with 0.25% aq. acetic acid. The eluates were lyophilized and an excess of anhydrous ethyl acetate was added to the residue to remove the non-volatile diethylene glycol. The material which precipitated was collected by centrifugation. After decanting the ethyl acetate the precipitate was again taken up in a few ml. of water + 1 drop of glacial acetic acid and finally lyophilized for storage and use.

3. *Bioassays for Pharmacological Activities of Various Materials*

The vasopressor and vasodepressor effects were studied by intravenous injection in dogs anesthetized with Nembutal with or without atropine. The more sensitive method of Dekanski (32) for vasopressin was also utilized with rats anesthetized with urethane and given dibenamine.

Oxytocic activity was tested on the virgin guinea pig uterus, in Tyrode solution at 33° C.

Gut-stimulating activity was checked on guinea pig or rabbit ileum in Tyrode at 37° C, (35 ml. chamber).

Antidiuretic activity was tested by intravenous injection in non-anesthetized hydrated dogs as described by van Dyke *et al.* (33).

Melanophoretic activity was estimated grossly in normal and hypophysectomized frogs (48 hours after hypophysectomy).

RESULTS

The chromatographic separation in solvent I of the methanolic extract of the hypothalamus, of Pitressin or of the methanolic extract of Protopituitrin gave a typical and constant picture of four main spots after ninhydrin spraying, labelled (and henceforth referred to as) fraction A, B, C, D, with mean Rf. values respectively of 0.06, 0.4, 0.7, 0.9. The fraction most active in releasing corticotrophin is fraction D (See Fig. 1, Tables 1 and 2). Fractions A and C have no ACTH-releasing activity, whereas fraction B showed some activity, both in hypothalamic (hog) or posterior pituitary material (Figure 1). Fraction D of hypothalamic origin appears to be more active on a weight basis than fraction D of posterior pituitary origin obtained by an identical procedure. Doses of 1 to 3 μg. of hypothalamic D (Table 2) gave potencies in stimulation of ACTH-release similar to those obtained with 100 μg. of posterior pituitary D (Table 1). At doses higher than 5 μg. hypothalamic D consistently produced inhibition of ACTH-release, a phenomenon never encountered with doses up to 250 μg. of

TABLE 1. ACTH-RELEASING ACTIVITY OF VARIOUS PREPARATIONS OF FRACTION D OF POSTERIOR PITUITARY ORIGIN

Protocol numbers	Addition	Potency M, % Std. with confidence limits at P = 0.05		Variance	λ
1986	10 μg.	125	150 104	3.4	0.042
1987	20 μg.	145	400 70	5.2	0.134
1988	50 μg.	242	484 159	6.4	0.083
2015	50 μg.	129	220 83	4.3	0.097
1898	100 μg.	272	678 108	15.6	0.146
1899	100 μg. + art.	174	226 139	4.0	0.046
1950	100 μg.	189	253 149	5.4	0.049
2056	100 μg.	243	448 165	8.4	0.080
2145	100 μg.	202	640 110	11.7	0.134
2193	100 μg.	246	466 166	7.2	0.080
2203	100 μg.	350	528 257	7.1	0.081
1914	200 μg.	240	340 180	5.2	0.050
2095	200 μg.	197	520 118	7.7	0.026
2119	200 μg.	211	1020 120	8.7	0.150
2122	200 μg.	525	748 308	13.0	0.150
2190	200 μg.	392	250 260	6.2	0.067

TABLE 1 (*continued*)

Protocol numbers	Addition	Potency M, % Std. with confidence limits at P = 0.05	Variance	λ
2192	200 μg.	480 (910 / 210)	4.4	0.064
2047	250 μg.	472 (1057 / 298)	5.1	0.080

Art. = 40 μg. dl-arterenol HCl.

posterior pituitary D. The explanation of this higher specific activity of the hypothalamic fraction as compared to the posterior pituitary material lies perhaps in the fact that the extraction procedure for peptides as described above, takes out a larger number of non-active substances of polypeptidic nature from the posterior lobe (34) than from the hypothalamus.

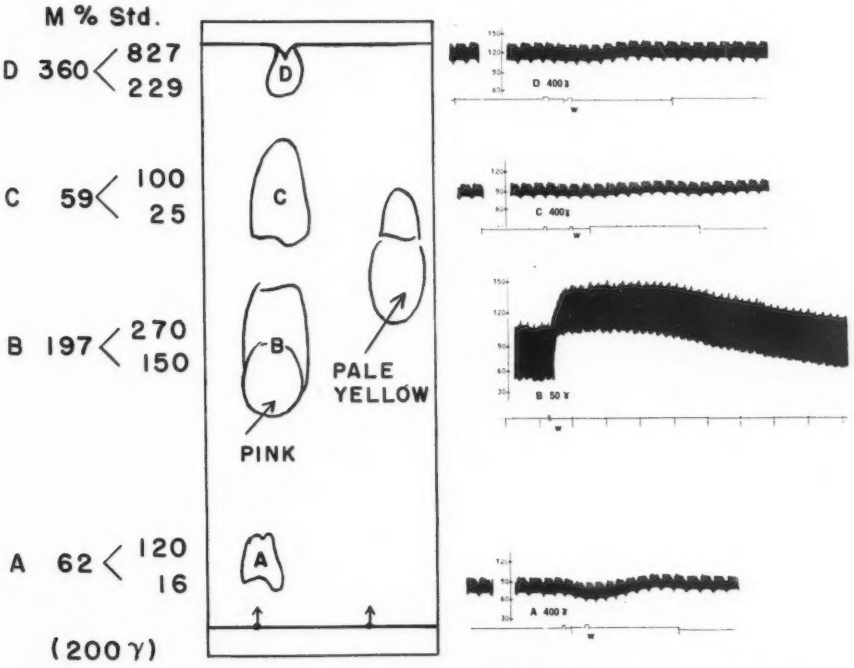

FIG. 1. From left to right: 1. Potency M, for ACTH-releasing activity, in % of standard, of the various chromatographic fractions A, B, C, D, of a methanolic extract of Protopituitrin in solvent I, shown in adjacent chromatogram. 2. Chromatogram in solvent I of a methanolic extract of Protopituitrin. At right, chromatogram of Pitocin in solvent I. 3. Vasomotor activity of the various fractions A, B, C, D; B is the only vasopressor fraction.

TABLE 2. ACTH-RELEASING ACTIVITY OF FRACTION D OF HYPOTHALAMIC ORIGIN

	Potency M, % Std.		Factorial analysis			Variance	λ
			Sample	Slope	Divergence		
200 μg.	6	95 4	S.	S.	N.S.	8.0	0.160
200 μg. +art.	34	52 16	S.	S.	N.S.	4.2	0.084
5 μg.	106	269 41	N.S.	S.	N.S.	14.2	0.149
2 μg.	169	228 132	S.	S.	N.S.	4.3	0.053
2 μg.	145	224 103	S.	S.	N.S.	6.9	0.076
2 μg.	139	191 106	S.	S.	N.S.	6.1	0.060
1.5 μg.	139	152 127	S.	S.	N.S.	1.3	0.019
3 μg.	142	215 101	S.	S.	N.S.	4.7	0.074
3 μg.	254	709 155	S.	S.	N.S.	5.7	0.111
1 μg. hog	145	219 104	S.	S.	N.S.	2.8	0.073
1 μg.	129	169 102	S.	S.	N.S.	3.9	0.053

Potency M, in % of standard with confidence limits of the assay at $p = 0.05$ calculated by analysis of variance.

Upper portion of Table = 0.5% acetic acid treatment at room temperature. Lower portion of Table = Kamm extraction, up to 95° C.

Tissues of bovine origin except lower two experiments (hog).

Art.: 40 μg. dl-arterenol HCl.

Rechromatography of fraction D of posterior pituitary and hypothalamic origin in another solvent confirmed this suggestion (see below).

As much as 200 μg. of fraction D of posterior pituitary origin did not show any evidence of corticotrophin contamination when assayed *in vitro* against U.S.P. Reference Standard for Corticotrophin (A-corticotrophin, in solvent I has an Rf. of 0.4). In contradistinction, 200 μg. of the methanol extract of Protopituitrin showed in the same test a contamination of 100 mU. of U.S.P. Corticotrophin per mg. Subsequently to this finding, batches

of 5 gm. of Protopituitrin were treated by two stirrings with 300 mg. of oxycellulose (Tennessee Eastman, 10% carboxyl) according to the method of Astwood *et al.* (35), which removed all detectable corticotrophic activity when tested in 200 μg. samples. The oxycellulose treatment did not appreciably alter yields of fraction D or its activity. The corticotrophin-releasing material contained in fraction D does not appear therefore to be adsorbed by oxycellulose in the conditions which remove ACTH.

The methanol extract of ACTH-free Protopituitrin and the purified fraction D were also tested with negative results for a possible potentiation of ACTH at the adrenal level. For this experiment, "native" rat ACTH was used to stimulate the adrenal fragments in preference to U.S.P. Reference Standard for Corticotrophin. This was achieved by using Krebs-Ringer bicarbonate medium in which rat anterior pituitary fragments had been incubated, as described above.

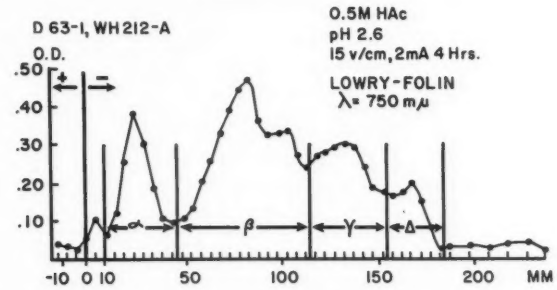

FIG. 2. Electrophoretic pattern of fraction D in 0.5 M acetic acid, pH 2.6, 15 v./cm., 2 mA, 4 hours.

When fraction D from posterior pituitary origin, obtained by chromatography in solvent I, was rechromatographed in butanol-acetic acid-water 4, 1, 5, or 4, 1, 1 (solvent II) in ascending paper chromatography (Whatman #1) for 12–14 hours, it showed four fractions, not too well separated (0.1 to 0.5); upon elution in 0.25% acetic acid and assay for ACTH-releasing activity of each area, inconclusive results were obtained, the activity being present all over the chromatogram. Electrophoresis of fraction D on paper (Whatman #3MM) in 0.5 M acetic acid, for four hours at 15 v./cm., 2 mA, showed several spots with ninhydrin, with an apparently similar distribution for fraction D of posterior pituitary or hypothalamic origin. In several different experiments, the electropherograms were cut every 5 mm., eluted in methanol and aliquots were read in a photometer after Folin-Lowry reaction (36). A typical curve is shown in Figure 2; a fast moving component (ε: 200, 250 mm.) often seen in various experiments is missing in this particular graph. The pattern indicated on Figure 2 was followed to cut electropherograms into strips for elution and recovery of the various peaks labelled α, β, γ, Δ, ε. When tested at 50 μg.

and 10 μg. level, in the *in vitro* assay for ACTH-releasing activity, none of the fractions was active, but fraction DΔ constantly produced inhibition of ACTH-release (i.e., potency M <1, in a statistically significant experiment) (Table 3). At 5 μg., results suggestive of stimulation of ACTH-release were obtained with DΔ alone. Amounts of 0.5 to 2.5 μg. of DΔ gave definite stimulation of ACTH-release, (see Table 3 for some representative results). With 6 anterior pituitary fragments weighing from 8 to 15 mg., these amounts per flask correspond to approximately 0.03 to 0.1 μg./mg. pituitary tissue stimulated. Negative results were obtained with the other fractions Dα, β, γ, ε, at any level studied. DΔ has no ACTH-potentiating effects when added (2 μg.) directly on the adrenal fragments stimulated with ACTH.

DΔ showed a positive reaction with ninhydrin, Pauly's reagent (37), Rydon and Smith chlorine+KI reaction for peptide bonds (38) and its modification by Reindal and Hoppe with benzidine (39), Folin-Lowry re-

TABLE 3. ACTH-RELEASING ACTIVITY OF ELECTROPHORETIC FRACTION DΔ

	Potency M, % Std.		Factorial analysis			Variance	λ
			Sample	Slope	Divergence		
50 μg. +art.	24	88 6	S.	S.	N.S.	11.3	0.172
10 μg. +art.	60	95 30	S.	S.	N.S.	7.8	0.101
5 μg. +art.	139	204 100	±S.	S.	N.S.	5.6	0.070
5 μg. +art.	198	290 150	S.	S.	N.S.	3.7	0.050
2.5 μg.	218	390 150	S.	S.	N.S.	5.1	0.083
2 μg.	144	172 123	S.	S.	N.S.	3.2	0.034
2 μg.	357	850 150	S.	S.	N.S.	5.6	0.117
2 μg.	169	228 132	S.	S.	N.S.	4.3	0.053
0.5 μg.	148	228 104	S.	S.	N.S.	5.1	0.077

Potency M, in % of Standard with confidence limits of the assay at P = 0.05 calculated by analysis of variance.
Art.: 40 μg. dl-arterenol HCl.

agent (36) and α-nitroso β-napthol (40). All these various reagents indicate presence of peptide bonds and peripheral amino-acid groups. When 300 μg. of DΔ were hydrolyzed and studied for amino acid content, 17 different amino acids were identified. DΔ therefore is still a mixture or a rather complicated peptide. Using solvent II, DΔ showed a single spot (after ninhydrin spraying) with an Rf. of 0.15–0.17, in an ascending chromatogram for 10–12 hours on Whatman #1 at room temperature.

The average Rf. of various amino acids chromatographed in solvent I where active fraction D has an Rf. of 0.9 are as follows: lys 0.05–0.11 (long spot but not streaked), arg 0.06–0.10 (same remark), γ-amino butyric ac. 0.42, his 0.24, cys 0.11, gly 0.27, gly-gly 0.24, glu 0.32, ala 0.41, try 0.53, tyr 0.53, phe 0.56, met 0.57, leu 0.60.

Stability of hypothalamic D, posterior pituitary D, and DΔ

Fraction D of posterior pituitary origin can be kept in boiling water for 15 minutes in an open tube in an aqueous neutral solution containing 1 mg./ml., without appreciable loss of activity in the *in vitro* assay. The activity is destroyed by refluxing after 60 minutes (pH 7.45) in boiling water. Fraction D of posterior pituitary origin has been kept for several months in the cold, as a lyophilized powder without detectable loss of activity. Three different batches, kept at room temperature as lyophilisates with no particular care lost activity in 2 to 3 weeks.

In contradistinction to the stability of crude fraction D, the more purified DΔ, approximately 100 times more active w/w, lost its activity within a few days, even when kept in the cold, in solution or in powder. The same observation was made with fraction D of hypothalamic origin. It is interesting to recall that hypothalamic D and DΔ have similar potency w/w; furthermore when fraction D of hypothalamic origin was rechromatographed in solvent II, it was observed that its main component (with ninhydrin) is a large spot having the same Rf. as DΔ of posterior pituitary origin in the same system (0.15–0.17). The hypothalamic material therefore appears to behave like highly purified DΔ and there is a suggestion that it might be made up essentially of this or a closely related substance.

The material which constitutes fraction D and the ACTH-releasing activity appear to be freely dialysable through a cellophane membrane. Several experiments were done to verify this point with dialysis in the cold (3–5° C) against water for 6, 12, 24 and 48 hours.

Control Tissues

Fragments of brain cortex (frontal, temporal, occipital lobes and cerebellum) of beef origin were treated according to the modification of the Kamm procedure described above in *2a*. After spraying with ninhydrin, chromatograms in solvent I of the methanol extract of these various tissues *never* showed the spot characteristic of fraction D as seen in posterior

pituitary or hypothalamic extracts prepared by the same method. As additional control studies, the methanol extract of one of the brain cortex fragments (frontal lobe) was tested for ACTH-releasing activity at a level which regularly gave positive results with methanol extracts of posterior lobe (100 μg.). No stimulation was observed; similarly negative results were obtained with 2 μg. of the eluate of the region: Rf. 0.8–0.99 of the frontal lobe chromatogram.

The classical Kamm method with heating in dilute acetic acid up to 95° C was also used to extract brain cortex. In contradistinction with the

TABLE 4. ACTH-RELEASING ACTIVITY OF BRAIN CORTEX—D (FRONTAL LOBE) BTAINED AFTER KAMM EXTRACTION (HOAc↗95° C)

	Potency M, % Std.		Factorial analysis			Variance	λ
			Sample	Slope	Divergence		
100 μg.	98	165 58	N.S.	S.	N.S.	9.7	0.096
2 μg.	180	347 117	S.	S.	N.S.	7.7	0.099
2 μg.	74	120 39	N.S.	S.	N.S.	7.9	0.101
2 μg.	114	184 74	N.S.	S.	N.S.	9.1	0.091

Potency M, in % of Standard with confidence limits of the assay at P = 0.05 calculated by analysis of variance.

results obtained after the mild extraction at room temperature, chromatographic separation of the methanol extracts of Kamm-treated brain cortex showed the characteristic spot D in solvent I. Although results were variable (see Table 4) in testing for ACTH-releasing activity, one assay gave definite stimulation of ACTH-release with 2 μg. of brain cortex D. When brain cortex D was rechromatographed in solvent II, its main component (with ninhydrin) appeared to have an Rf. of 0.15–0.17 which is the mobility coefficient of DΔ of posterior pituitary origin.

Substance P

Two samples[6] of substance P of gut origin showed the characteristic D spot upon chromatography in solvent I. Both crude preparations of substance P stimulated release of ACTH *in vitro* (Table 5). Fraction D of the substance P sample of horse gut origin stimulated ACTH-release at levels similar to what was observed for the hypothalamic material or DΔ (Table

[6] 13.8 Euler U./mg. (horse gut) and 275 Euler U./mg. (cow gut) kindly sent by Dr. M. Vogt and Dr. B. Pernow respectively.

5) and inhibited release of ACTH when added at levels exceeding 10 μg. Fraction D from this sample of substance P, when rechromatographed in solvent II, showed (with ninhydrin) four spots and *again* as the main component a fraction with the 0.15–0.17 Rf. A preparation of substance P of brain origin (beef) made in our laboratory (9.5 Euler U./mg.) showed the classical D fraction upon chromatography in solvent I. No conclusive re-

TABLE 5. ACTH-RELEASING ACTIVITY IN VARIOUS PREPARATIONS OF SUBSTANCE P

	Potency M, % Std.		Factorial analysis			Variance	λ
			Sample	Slope	Divergence		
Subst. P* 5 μg.	136	178 107	S.	S.	N.S.	5.4	0.038
Subst. P* 10 μg.	129	156 108	S.	S.	N.S.	2.5	0.130
Subst. P** 10 μg.	201	636 117	S.	S.	N.S.	9.5	0.050
P* fract. D 20 μg. +art.	115	133 99	N.S.	S.	N.S.	2.9	0.032
P* fract. D 5 μg.	160	210 127	S.	S.	N.S.	2.0	0.050
P* fract. D 2.5 μg.	173	204 150	S.	S.	N.S.	2.5	0.030

Potency M, in % of Standard with confidence limits of the assay at P = 0.05 calculated by analysis of variance.
* Substance P: 13.8 Euler U./mg., horse gut. P* fraction D, in solvent I.
** Substance P: 275 Euler U./mg., cow gut.
Art.: 40 μg. dl-arterenol HCl.

sults were obtained regarding stimulation of ACTH-release with this crude preparation. Swingle *et al.* (49) have recently presented data interpreted as evidence of stimulation by Substance P of ACTH-release *in vitro*.

Yields

Typical yields are as follows:

Posterior pituitary (beef) acetone powder, 14.3 gm.→1 gm. Protopituitrin→methanol extract, 373 mg.→69.3 mg. fraction D, i.e., 0.48% of acetone powder.

Protopituitrin (hog): 10 gm. Protopituitrin→3.5 gm. methanol extract →525 mg. fraction D, i.e., 5% of Protopituitrin.

Posterior pituitary (hog) fraction D: 48.3 mg.→ca. 1.0 mg. DΔ, i.e., 0.001% of Protopituitrin.

Hypothalamus (hog): acetone powder 7 gm.→Kamm extraction,

→methanol extract of Kamm's end product 32.5 mg.→7.7 mg. fraction D, i.e., 0.11% of acetone powder.

Hypothalamus (beef): acetone powder 20.6 gm.→Kamm extraction, →methanol extract of Kamm's end product 61.8 mg.→4.8 mg. fraction D, i.e., 0.02% of acetone powder.

Brain cortex (beef): acetone powder 18.2 gm.→Kamm extraction, →methanol extract of Kamm's end product 56.8→8.9 mg. fraction D, i.e., 0.04% of acetone powder.

The exact meaning of these various figures is not clear at the present time, since the end products of the same procedures carried out on different tissues, differ in purity and activity depending on the tissue of origin (e.g., fraction D from hypothalamus is more active w/w than the material extracted from the posterior pituitary).

Effects of Arterenol

In contradistinction to the reports of Saffran *et al.* (15, 26) we could never observe any sign of a permissive or of a conditioning effect of arterenol for the ACTH-hypophysiotropic activity of any of the material isolated, whether of posterior pituitary or of hypothalamic origin as can be seen in Tables 1, 2, 3, 4, and 5.

From the *pharmacological studies*, area B of posterior pituitary or hypothalamic origin appears to be the vasopressin containing fraction (Fig. 1). Fraction B of *hypothalamic* origin may also contain a vasodepressor material which sometimes interferred with the pressor assays; a similar observation has been made by van Dyke *et al.* (33) on hypothalamic extracts. Fractions A, C, and D did not show any vasopressor activity when tested in the dog (Fig. 1). In assays against U.S.P. Posterior Lobe Reference Standard in the rat treated with dibenamine, several batches of fraction D of posterior pituitary origin showed a minimal but definite vasopressor activity (4 to 50 mU./mg.). Fractions D of hog or ox hypothalamus had no vasopressor effect in the dog or the rat assay. When assayed for antidiuretic activity in the non anesthetized hydrated dog, posterior pituitary fraction D contained 43 mU./mg. while a sample of beef hypothalamus fraction D did not exhibit any activity.

Fraction D prepared from the sample of substance P received from Dr. M. Vogt had none of the pharmacological characteristics of substance P (i.e., gut stimulating activity, vasodepressor effects) which could readily be demonstrated with the original sample of substance P.

Fraction D from hypothalamus or posterior pituitary (up to 500 μg.) has no histamine-like activity on the blood pressure of the dog or of the rat nor on the isolated gut (guinea pig ileum or rabbit ileum; sensitivity of the preparations $\leqq 1$ μg. histamine). This material therefore is not similar to the "histamine-like" fraction isolated from Pitressin and reported by Swingle *et al.* (41) as active in releasing ACTH *in vitro*. The absence of smooth-muscle stimulation on the isolated gut is also evidence against

identity of fraction D and serotonin (sensitivity of the preparation $\leqq 0.4$ μg. serotonin creatinine sulfate).

The results of the pharmacological characteristics of fraction D are consistent with the view that we are not dealing with histamine, adrenaline, arterenol, acetylcholine, serotonin (5-hydroxytryptamine), vasopressin, or "true" substance P.

The oxytocin assays with the isolated strips of guinea pig uterus were inconsistent and showed negligible activity over the entire surface of the chromatograms.

When fraction D of posterior pituitary (500 μg.) or *hypothalamic* (200 μg.) origin was injected in the dorsal lymph sac of normal and hypophysectomized frogs, there was a rapid ($\leqq 60$ minutes) darkening of both groups of animals. Subsequently these frogs showed a considerably lighter color than non-injected frogs having been in the same lighting conditions. It was further noted that the frogs previously injected with fraction D of posterior pituitary or hypothalamic origin took considerably more time to darken upon a second injection 24 hours later, than otherwise normal frogs. This fact and the intense blanching following the initial darkening phase suggest the presence of two factors, a melanophore stimulating factor (MSH ?) and an antagonist, or melanophore contracting factor (anti-MSH ?), in the rather crude fraction D.

DISCUSSION

A discussion of these results calls for an appraisal of the various methods utilized in this series of *in vitro* experiments.

a) *Assessment of adrenocorticotrophic activity*

There is ample evidence (29, 42, 43, 44, 45, 46, 47) that the *in vitro* corticoidogenesis of the adrenal fragments is adequately measured by the U.V. absorption at 240 mμ of the methylene chloride extract of their incubation fluid. This corticoidogenesis appears to be specifically influenced by ACTH (29, 44, 45). With U.S.P. Reference Standard for Corticotrophin and various commercial preparations of ACTH, results in agreement with those of others have consistently been obtained in this laboratory. Although the point was not extensively studied, stimulation of corticoidogenesis was never encountered with materials that were not supposed to contain ACTH, such as water, gelatin USP, purified extracts of the hypothalamus, of the brain cortex or of the posterior pituitary.

b) *Stimulation of ACTH-release*

The design of the assay for ACTH-hypophysiotropic activity (see page 489) implies that there is no discrepancy in the amounts of corticotrophin released by each half anterior pituitary. A large number of assays for ACTH-release by half pituitaries as tested against their other halves and assays of ACTH against itself always gave a ratio of *one* (Tables 6 and 7),

with not statistically significant variations around unity depending on the precision of the assay. Evidence has already been presented above, that the results reported in Tables 1, 2 and 3 were neither due to a direct adrenocorticotrophic effect of the various fractions studied nor to a potentiation of corticotrophin at the adrenal level. If inactivation of the released corticotrophin takes place during the pituitary incubation (a problem which we did not investigate) it should be similar in both beakers for both half pituitaries and should not alter the results. That it is so, is perhaps best

TABLE 6. CONTROL STUDIES ON PERFORMANCE OF THE ASSAY FOR ACTH-RELEASING ACTIVITY BY TESTING EACH HALF PITUITARY AGAINST ITS OTHER HALF

Protocol numbers		Potency M with confidence limits at P = .05	Variance	λ
1968	½ Pit. vs. ½ Pit.	0.796 1.109 0.596	4.3	0.072
2173	½ Pit. vs. ½ Pit.	1.05 1.600 0.700	4.6	0.074
2176	½ Pit. vs. ½ Pit.	1.03 1.52 0.71	9.1	0.078
2180	½ Pit. vs. ½ Pit.	1.03 1.680 0.640	7.3	0.096
2183	½ Pit. vs. ½ Pit.	1.24 1.65 1.08	4.1	0.060
2184	½ Pit. vs. ½ Pit.	0.89 1.11 0.71	5.3	0.045
2186	½ Pit. vs. ½ Pit.	0.966 1.32 0.81	4.4	0.051
2189	½ Pit. vs. ½ Pit.	0.935 1.27 0.89	1.9	0.042
2197	½ Pit. vs. ½ Pit.	0.79 2.31 0.77	8.1	0.111
2198	½ Pit. vs. ½ Pit.	1.35 2.61 0.81	9.5	0.106
2199	½ Pit. vs. ½ Pit.	0.966 2.6 0.91	4.6	0.108
2200	½ Pit. vs. ½ Pit.	0.98 1.32 0.78	4 4	0.057

Mean Potency for 12 Experiments, ±Standard Error: M = 1.00 ±0.31.

TABLE 7. CONTROL STUDIES ON PERFORMANCE OF THE ASSAY FOR ACTH-RELEASING ACTIVITY BY TESTING U.S.P. REFERENCE STANDARD FOR CORTICOTROPHIN AGAINST ITSELF AT SIMILAR DOSES AND BY TESTING NATIVE RAT ACTH AGAINST ITSELF

Protocol numbers		Potency M, with confidence limits at P = 0.05		Variance	λ
2075	ACTH USP*	1.026	2.128 0.506	9.5	0.128
2076	ACTH USP	0.748	1.93 0.123	14.0	0.184
2077	ACTH USP	1.56	2.90 1.00	8.8	0.097
2078	RAT ACTH**	0.89	1.93 0.36	14.0	0.143
2079	RAT ACTH	0.83	1.4 0.42	9.4	0.114

* 10 mU/100 mg. of adrenals as S_1, U_1; 30 mU/100 mg. adrenals as S_2, U_2.
** Native rat ACTH obtained by grinding 8 half pituitaries in 3 ml. Krebs-Ringer bicarbonate added with 0.1 ml. of 1 N, HCl. The tube is centrifuged at room temperature (2000 RPM for 5 min.) and kept in ice until use, 60 minutes later. After neutralization with 0.1 ml. of 1 N, CO_3HNa, (Sol. A), 1 ml. of Sol. A is diluted to 3 ml. with Krebs-Ringer (Sol. B). Aliquots of 0.1 ml. of Sol. A and Sol. B are used as S1, U1, and S_2, U_2 in the assay.

exemplified by the potency ratios of one obtained when halves of the same pituitaries were tested against each other (Table 6) or when identical amounts of ACTH were similarly tested (Table 7). From this analysis it seems therefore that the results reported are consistent with "stimulation of ACTH-release." No explanation is offered at the moment for the results showing "inhibition" of ACTH-release with doses of DΔ or hypothalamic D larger than 5 μg. This response was not altered by presence or absence of arterenol.

c) *Specificity of the in vitro hypophysiotropic activity*

This is a question of major interest in view of the well known non-specificity of the procedures which *in vivo* will stimulate ACTH-release. Of all the chromatographic fractions tested, only fraction D and the vasopressin-containing fraction[7] in hypothalamus or posterior pituitary tissues have stimulated release of ACTH in this test, *in the conditions used in this laboratory and described above for these assays.* Synthetic oxytocin (0.2 to 5 μg.), histamine (1 and 2 μg.), arterenol (40 μg.), gelatin USP (1 mg.) were

[7] The question of the activity of the molecule of vasopressin as such and as a component of the vasopressor fraction B is still being investigated and will be reported separately. It may be of interest however to mention here that a preparation of arginine vasopressin obtained from Dr. Du Vigneaud showed in solvent II in addition to vasopressin a spot (with ninhydrin) having the same Rf. (0.15–0.17) as DΔ.

also assayed and found inactive in stimulating ACTH-release. Whereas stimulation of ACTH-release in this system was not limited to hypothalamic tissue, a single common chromatographic fraction (fraction D)[7] seems however to have been responsible for this stimulation. Its extractability with mild procedures suggests that this material in the hypothalamus and the posterior pituitary is in a more labile state than in any other tissue where more radical extraction methods were necessary to detect its presence. Identical chromatographic behavior of otherwise active materials in only two different solvents, (especially when in one of these the Rf. is close to 1.0) is not enough to conclude that these materials are identical. Thus it cannot be ascertained whether or not the "crude" fractions D extracted from hypothalamus, posterior pituitary, brain cortex or intestine are similar. We also cannot conclude whether their active component is a unique substance (apparently identical to DΔ). The possibility that the stimulation for ACTH-release of the *in vitro* pituitary might be obtained with several molecules of a family of closely related peptides is not excluded on the basis of the results presented here.

With all these important restrictions in mind, if we assume that the *in vitro* results may apply *in vivo*, they would lead to interesting speculations regarding the theories of a biphasic or of a multiple control for ACTH-release. The ACTH-releasing principle would be a particular substance or structure with a wide distribution pattern in the organism. A special though not exclusive role would be attributed to the hypothalamus because of its unique anatomical neurovascular relationship with the pars distalis and because of the results suggesting a greater lability of the ACTH-hypophysiotropic material in this region. This characteristic would make this material more readily available for pituitary stimulation, when distributed through the hypophysial parenchyma via the ramifications of the portal vessels. This would be in agreement with the numerous results involving the hypothalamus in stimulation of ACTH-release. The ACTH-hypophysiotropic material of extra-hypothalamic origin released from various tissues by a non-specific stress and delivered through the general circulation would explain the release of corticotrophin which can still take place under severe nonspecific stress after severance of the normal hypothalamo-pituitary relationship or destruction of median eminence. The fact that this response, though significant, is nevertheless of lesser amplitude than the one obtained when hypothalamo-pituitary connections are intact might have to be related to some resistance to stimulation of both the isolated anterior lobe and the adrenal cortex (cf. the atrophy of the anterior pituitary (19) and adrenals following such procedures). It would be of great interest to study the pattern of ACTH-release obtained in such preparations with chronic administration of the material of hypothalamic origin. Although the data reported above suggest that the posterior lobe of the pituitary may play some part in ACTH-release, they do not clarify whether or not it is under hypothalamic control.

A critical appraisal of the physiological meaning of all these results obtained *in vitro* appears to be necessary: their true significance, if any, will have to be assessed in some dynamic fashion *in vivo* lest they remain of purely esthetic value.

SUMMARY

A substance has been isolated from the hypothalamus and the posterior pituitary (hog and beef tissues) which stimulates release of ACTH from rat pituitary tissue *in vitro*. The purified material appears to be a small peptide, different from vasopressin, oxytocin, ACTH, histamine, acetylcholine, adrenalin, nor-adrenalin and 5-hydroxytryptamine by its pharmacological and chromatographic characteristics. The *in vitro* ACTH-releasing activity was also observed in a fraction of Substance P of gut origin and possibly in extracts of brain cortex. In all cases the activity appears to have been located in a common fraction (Fraction D). It is felt that the physiological significance of these *in vitro* results should be assessed *in vivo*.

Acknowledgments

We are pleased to acknowledge the excellent assistance of Miss S. Williams, M.Sc. We are indebted to Dr. D. A. McGinty, Parke, Davis and Co., Dr. S. Steelman, Armour Laboratories, Dr. K. M. Smoot, Winthrop Laboratories, Dr. R. Bircher, Sandoz Laboratories, and Dr. M. McCormick, Lilly Research Laboratories, for having furnished us with various starting material or hormonal preparations.

We also wish to express our gratitude to Dr. J. Awapara (Department of Biochemistry, M.D. Anderson Hospital, University of Texas, Houston, Texas) for having studied the amino acid composition of a hydrolysate of fraction DΔ and to Dr. S. Engel (Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, N. Y.) for having assayed several of our fractions for antidiuretic activity.

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EFFECT OF CORTICOIDS UPON EXPERIMENTAL LATHYRISM

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LATHYRISM, a disease due to ingestion of excessive amounts of sweet pea seeds (*Lathyrus odoratus*) or allied species of lathyrus, has been known since the time of Hippocrates. Now, the malady is particularly prevalent in certain parts of India and Spain, where lathyrus seeds are commonly used as an inexpensive source of nourishment. It has also long been established that a form of lathyrism can be produced at will in various laboratory animals (rats, rabbits, guinea pigs, monekys, etc.) by feeding them sweet pea diets. Among the changes most characteristic of experimental lathyrism are: deformities of the bones, with multiple exostoses, hernias and dissecting aneurysms. The earlier literature on lathyrism in man and in experimental animals has been reviewed by Stockman (13) and Geiger *et al.* (4).

Recently, interest in this singular condition has been greatly stimulated by the demonstration that the active principle, or principles, can be extracted from the sweet pea seeds (7), and that certain nitriles, particularly β-aminopropionitrile, present in Lathyrus seeds, can reproduce the disease in animals (11, 14, 2). Aminoacetonitrile (AAN) is even more effective than β-aminopropionitrile in producing lesions characteristic of lathyrism in mesenchymal tissues (14, 10).

A great deal of work has been done, in an effort to prevent experimental lathyrism, with a variety of agents, but the relevant literature is highly confusing. It had been claimed that α-tocopherol (6) and casein (1) confer some protection, but more recent experiments led to the conclusion that neither these nor most of the other compounds tested (e.g. gelatin, pantothenic acid, glucosamine, a great variety of drugs and food supplements) can effectively prevent this condition (2, 3, 5, 8). It would appear from all available data that experimental lathyrism is singularly resistant to treatment, although allegedly L-cysteine-HCl, L-glutamine, and certain proteins are partially protective (3). The effect of hormones has not yet been systematically investigated, but testosterone was shown to be incapable of preventing experimental lathyrism in the rat (9).

In view of our interest in the effect of hormones upon mesenchymal tissue reactions we have performed a series of orientating experiments with a large number of hormone preparations in rats maintained on a 50% *L.*

Received October 8, 1956.

odoratus and 50% "Purina Fox Chow" diet. The principal result of these investigations was to show that cortisol acetate (COL-Ac) inhibits, while somatotrophic hormone (STH) aggravates, the skeletal deformities. It was observed furthermore, that in rats sensitized to mineralocorticoid actions (by unilateral nephrectomy and salt supplements) desoxycorticosterone acetate (DOC-Ac) produces aortic aneurysms and ruptures with extraordinary frequency (12).

Since, in the form of AAN we now possess a rather specific poison for certain mesenchymal tissues, it was felt that these experiments should be repeated and extended with this drug. It is the object of this communication to show that under suitable experimental conditions, it is possible to suppress virtually all the skeletal deformities caused by AAN, while DOC-Ac can selectively sensitize the aorta to the production of aneurysms by this compound.

EXPERIMENTAL MATERIAL AND TECHNIQUES

60 female Sprague-Dawley rats having a mean initial body weight of 100 gm. (range 95–108 gm.) were subdivided into six equal groups. All animals received 5 mg. of AAN in 0.2 ml. of water subcutaneously, once daily during the first three days of the experiment, and twice daily until the end of the experiment. In order to establish the possible effect upon experimental lathyrism of the "conditioning" procedures usually employed to increase sensitivity to mineralocorticoids, the right kidney was removed (through a subcostal incision under ether anesthesia) on the first day in Groups 2–6, and a 1% NaCl solution was substituted for tap water as drinking fluid in Groups 3–6. Both DOC-Ac and COL-Ac were administered as microcrystal suspensions at the dose level of 1 mg. in 0.2 ml. of aqueous suspension fluid, once daily subcutaneously.

On the 20th day the animals were killed with chloroform. The degree of skeletal lathyrism was then estimated on an arbitrary scale of 1–3, as judged by the intensity of the macroscopically visible bone deformation of the ribs. Lesions in these bones proved to be convenient indices of the skeletal affection because they are inspected easily from the inside of the thorax, underneath the pleural covering. We arrived at the mean degree of lathyrism by dividing the total score by the number of rats in each group. This mean is listed in Table 1, together with the final body weight (in gm.), the gain (gm. per 100 gm. of body weight) and the percentile incidence of aortal aneurysms.

For the purpose of histologic study, the left knee joint was fixed (and at the same time decalcified) in Susa solution, embedded in paraffin and stained with hematoxylin eosin. For better appraisal of the extent of skeletal lathyrism, some of the other bones—especially the frequently affected spinal columns and femurs—were also dissected. Special attention was paid to the anterior tubercle of the axis, which we found to be the most regularly and intensely affected.

RESULTS

Our principal findings are summarized in Table 1.

In the first three groups, in which no corticoid treatment was given, all the rats developed marked skeletal lathyrism, but no aortal aneurysms. Apparently unilateral nephrectomy, alone or in combination with NaCl supplements, did not markedly affect the course of AAN intoxication. On the other hand, among the rats of Group 4 which were treated with DOC-Ac—following conditioning by unilateral nephrectomy and NaCl—the

TABLE 1

Group	Corticoid treatment	Conditioning*	Final body wt. (gm.)	Gain (%)	Lathyrism of ribs	Aortal aneurysms (%)
1	None	None	128	+28	1.5	0
2	None	KX	105	+ 5	2.2	0
3	None	KX+NaCl	112	+12	1.9	0
4	DOC-Ac	KX+NaCl	121	+21	0.8	20
5	COL-Ac	KX+NaCl	99	− 1	0	0
6	DOC-Ac+ COL-Ac	KX+NaCl	106	+ 7	0	30

* KX = Right kidney extirpated, NaCl = 1% NaCl given as drinking fluid.

skeletal lesions were somewhat less pronounced than in the animals given no corticoid treatment; however, two animals in Group 4 developed aortal aneurysms. The COL-Ac treated rats of Group 5 exhibited no macroscopically detectable evidence of skeletal lathyrism and no aortal aneurysms. Among the rats of Group 6, which received both DOC-Ac and COL-Ac, there was a 30% incidence of aortal aneurysms and yet no detectable skeletal deformity.

These changes are illustrated in Figures 1–9. The inhibition of skeletal lathyrism by COL-Ac is particularly obvious in those bones which normally develop the most pronounced exostoses and deformities during AAN treatment. For instance, the anterior tubercle of the axis (Fig. 1), the coronoid and condyloid processes of the mandible (Fig. 2) and the muscle insertion sites on the femur (Figs. 3 and 4) were enormously enlarged and deformed in all the AAN treated rats, which received no COL-Ac treatment. No such changes were observed in the corresponding skeletal parts of animals given COL-Ac, either alone, or in combination with DOC-Ac.

Histologic study of the affected bones showed a development of excessive, partly uncalcified, bone tissue at muscle insertion sites and underneath the periosteum throughout the skeleton. In the epiphyseal cartilages (for instance in the distal epiphysis of the femur) the chondrocytes were greatly enlarged and many of them were in mitotic division. The normal columnar arrangement of cartilage cells in the zone of primary calcification was almost totally lost. The intercellular cartilage matrix underwent degeneration and became eosinophilic; it also became tingible with the PAS technique. In the middle of the junction cartilage plate the degenerative process went so far as to cause a cleft filled by totally liquefied, degenerated tissue (Figs. 5 and 7). Often, the irregular proliferation of regenerating cartilage cells exhibited a chondroma like appearance. Islets of such tissue tended to become split from the original cartilage plate and to form isolated accumulations between the bony spicules of the epiphysis or diaphysis (Figs. 6 and 8). All these changes were virtually absent in the bones of rats treated with COL-Ac, although in some animals, occasional irregularities in cartilage or bone structure could be demonstrated upon careful, histologic investigation.

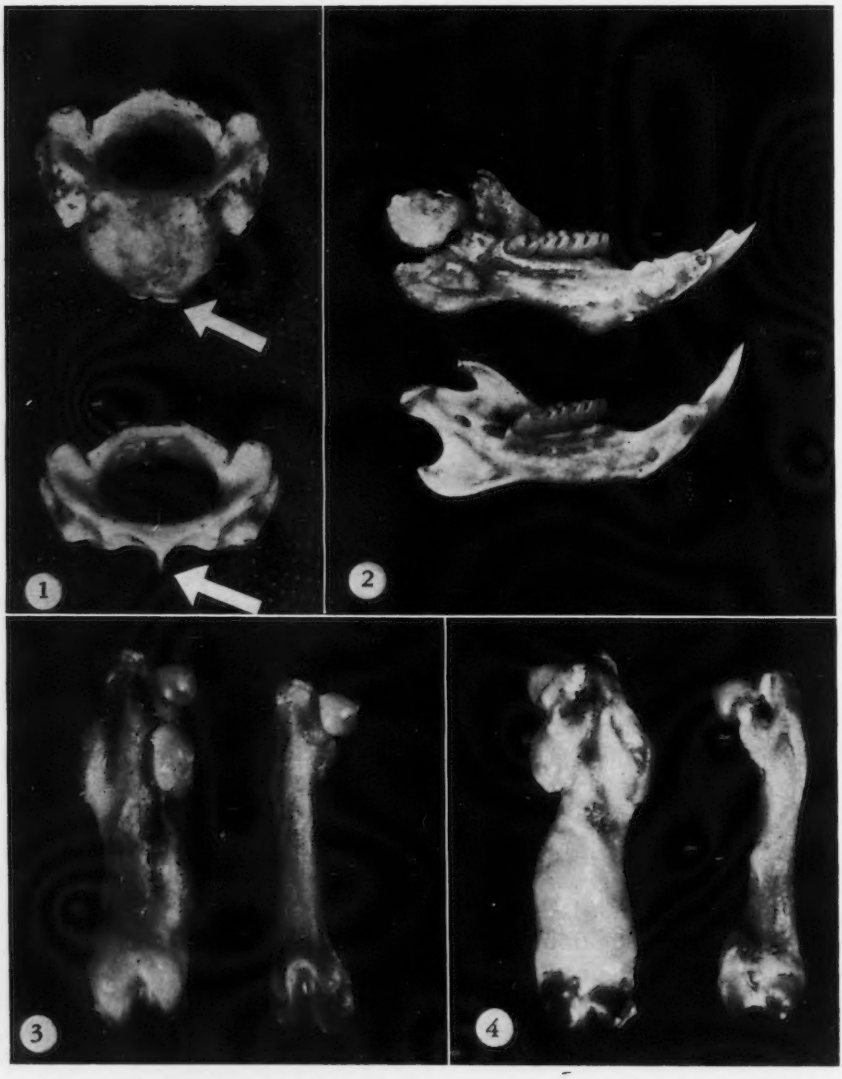

FIG. 1. Axis (cranial view) following treatment with AAN alone (top) and AAN +COL-Ac (bottom). The excessive bone proliferation with the development of exostoses, especially at the site of muscle insertions, is inhibited by the antiphlogistic corticoid. This inhibition is especially evident, as regards the enormous exostosis that always develops at the site of the anterior tubercle of the axis (arrow).

FIG. 2. Left mandible (buccal view). Voluminous exostoses, at muscle insertions—particularly obvious at the coronoid and condyloid processes and at the angle of the jaw —in a rat receiving AAN alone (top), while the bone structure remains essentially normal in the animal given AAN+COL-Ac (bottom). Note also abnormal configuration of the incisor tooth in the rat receiving no hormone treatment.

FIG. 3. Right femur (anterior view) and FIG. 4. (posterior view). Here again the intense periostal bone formation and the exostoses which develop at the site of muscle insertions in the rat treated with AAN alone (left) are virtually absent in the animal given concurrent treatment with COL-Ac (right).

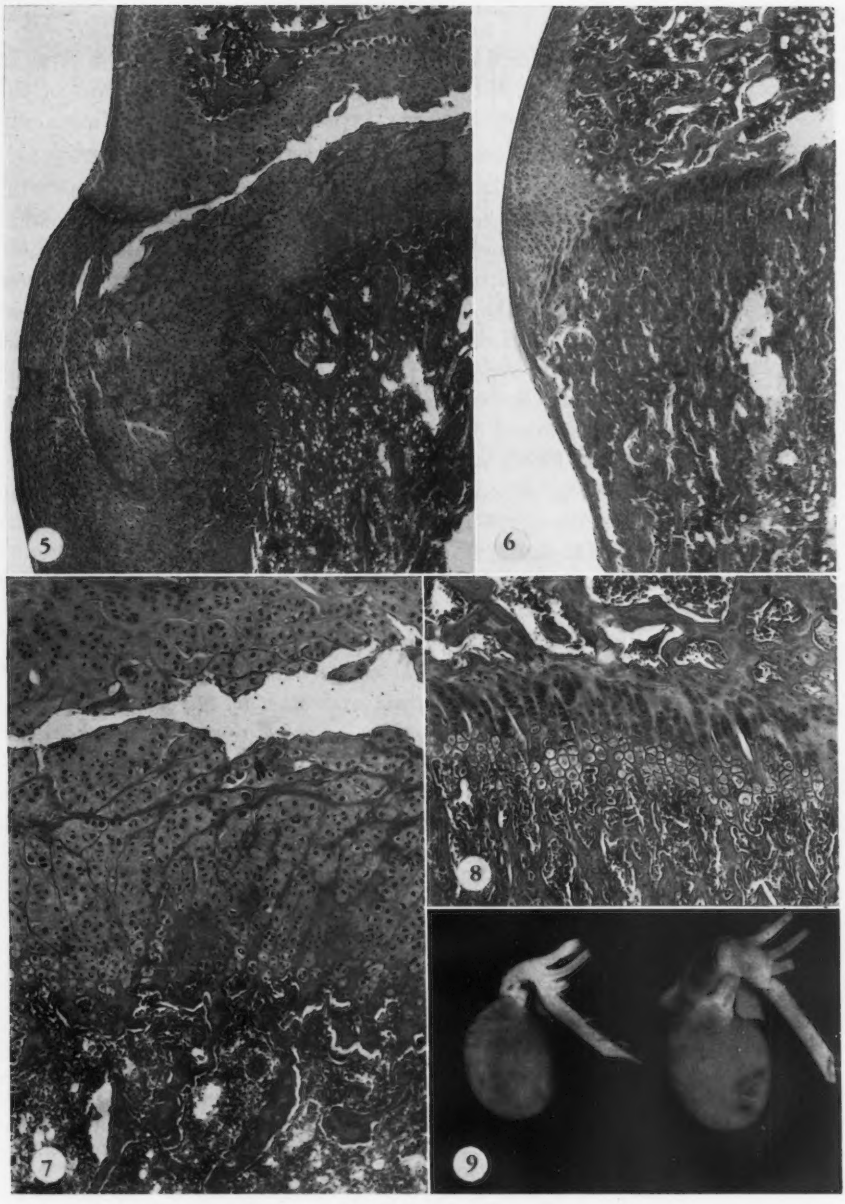

FIG. 5. Histologic section through the epiphysial cartilage region of the lower end of the femur in a rat of Group 3. The cartilage plate is greatly thickened, and a wide cleft has developed in its center. There is extensive proliferation of only partially calcified or totally uncalcified bone near the lower left corner of the field.

FIG. 6. Corresponding bone region of a rat from Group 5. COL-Ac treatment virtually suppresses all the osseous manifestations of experimental lathyrism.

(*Legends for Figs 7, 8 and 9 on following page*)

The aneurysms in Groups 4 and 6 were always situated on the cross of the aorta. Macroscopically, they appeared as deep red dilatations of the vessel, and histologically they bore the typical aspect of dissecting aneurysms (Fig. 9).

DISCUSSION

It is evident, from these observations, that the development of skeletal lathyrism under the influence of AAN can be prevented by suitable glucocorticoid treatment. Since neither the cartilaginous nor the bony proliferations are inflammatory, in the usual sense of the word, this observation confirms our view, that *corticoids can influence tissue reactivity to various agents and not only to those which elicit typical inflammatory responses.* Our findings also suggest that the blood level of corticoids can be the determining factor in the development of a skeletal disease that is due to an exogenous poison. Whether DOC-Ac can also exert a similar suppressive effect upon skeletal lathyrism remains undecided. Although in Group 4 the bone lesions were less pronounced than in the first three groups, this difference was of doubtful significance.[1] However, it is clear that under our experimental conditions at least, DOC-Ac did not prevent the prophylactic action of COL-Ac, so that in this respect there was no demonstrable antagonism between a mineralo- and a glucocorticoid hormone.

It is also noteworthy that *the individual morbid changes, characteristic of the malady, can be totally dissociated from each other by merely changing the mineralo- or glucocorticoid level of the milieu in which an experimental disease develops.* Thus, in the present experiments, AAN produced extensive skeletal lesions without aortal aneurysms in otherwise untreated rats; DOC-Ac failed to suppress the skeletal lesions, but made it possible for the AAN to elicit aortal aneurysms, while combined treatment with DOC-Ac and COL-Ac completely altered the character of the disease, in that skeletal deformities were absent and yet aortal aneurysms developed, at least in some of the animals. It may be added, that in our earlier work on lathyrism (12) induced with diets containing 50% *L. odoratus* seeds, treatment with DOC-Ac (following conditioning by unilateral nephrectomy and NaCl)

[1] Possibly here DOC-Ac overdosage acted through the stimulation of endogenous glucocorticoid secretion elicited by the non-specific damaging effect of this steroid. This has not been proven but hitherto unpublished experiments revealed that, in adrenalectomized rats, DOC-Ac fails to suppress the skeletal changes normally produced by AAN.

FIG. 7. Higher magnification of a field from the bone shown in Figure 5. Here the great enlargement of the cartilage cells, the loss of columnar architecture and the degeneration of the matrix with cleft formation are particularly obvious.

FIG. 8. High magnification of a field from Figure 6. Normal columnar arrangement of the cartilage cells in the epiphysial plate and essentially normal bone structure.

FIG. 9. Large dissecting aneurysm in a rat treated with DOC-Ac (after conditioning for mineralocorticoids by unilateral nephrectomy and initial supplements). Compare large dissecting aneurysm in the cross of the aorta (right) with the normal aorta of a similarly conditioned and AAN treated rat which received no DOC-Ac.

produced aortal aneurysms in almost every animal, although control rats, kept on the same diet, developed aneurysms only in exceptional instances.

SUMMARY

Experiments are described in which aminoacetonitrile (AAN) produced pronounced skeletal deformities, but no aortal aneurysms in the rat.

Under these same conditions, treatment with cortisol acetate (COL-Ac) suppressed the skeletal lesions but did not sensitize for the production of aortal aneurysms.

Conversely, treatment with desoxycorticosterone acetate (DOC-Ac) exerted no marked effect upon skeletal lathyrism, but sensitized the rat to the production of aortal aneurysms by AAN.

Finally, combined treatment with DOC-Ac and COL-Ac completely reversed the normal morphologic syndrome of experimental lathyrism, in that this corticoid combination caused AAN to produce aortal aneurysms in the virtual absence of bone lesions.

These observations furnish additional support for the concept that the corticoids can participate in the development of the most diverse non-endocrine diseases. As shown by the example of experimental lathyrism, these hormones can selectively suppress some, and exacerbate other, manifestations of morbid processes. Emphasis is laid upon the fact that this is true even of non-inflammatory tissue reactions to an exogenous irritant.

Acknowledgments

This work was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the United States Army, Contract No. DA-49-007-Md 186.

The authors wish to thank the Schering Corporation for supplying desoxycorticosterone acetate ("Cortate"), the Pfizer Laboratories for cortisol acetate ("Cortril") and Dr. I. V. Ponseti of Iowa State University for aminoacetonitrile hydrosulfate.

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HEPATIC LIPOGENESIS IN THE DIABETIC-HYPOPHYSECTOMIZED (HOUSSAY) RAT[1]

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IT WAS demonstrated earlier that, provided carbohydrate in the form of glucose or starch is available in large amounts to hypophysectomized rats, hepatic lipogenesis, as judged by the conversion of acetate carbon to fatty acids, does not differ significantly from that observed in normal rats (1). Our findings offered no support for the view that hepatic lipogenesis is accelerated in the absence of pituitary hormones (2, 3, 4). Indeed, when hypophysectomized rats were fed a stock diet adequate in all respects, but containing whole ground wheat as the major carbohydrate source, lipogenesis and cholesterogenesis in their livers were below normal (1). The results obtained by Hill *et al.* (1) emphasize the need for a careful evaluation of the nutritional state of the hypophysectomized animal before ascribing a metabolic effect to the removal of the anterior pituitary gland.

It is now generally agreed that the diabetic animal has lost, to a considerable extent, its capacity to form fat from carbohydrate (5, 6). A number of investigators have demonstrated, in recent years, that the incorporation of C^{14}-labeled acetate into fatty acids by liver slices from alloxan-diabetic rats is depressed (7, 8). In order to explore further the role of the anterior pituitary gland in lipogenesis, we have studied here the conversion of acetate to fatty acids by livers of rats first made diabetic and then deprived of their pituitary glands (Houssay rats).

EXPERIMENTAL

Male rats of the Long-Evans strain were used. Diabetes was induced by a single intravenous injection of 50 mg. of freshly recrystallized alloxan monohydrate (Eastman) per kg. of body weight. Daily records were kept of food consumption of all rats. In the case of diabetic rats, the urine volume and glucose output in the urine were also measured daily. Only those diabetic rats that manifested a blood sugar in excess of 200 mg. % after a 10-hour fast were chosen for study. At least 30 days were allowed to elapse, after the injection of the alloxan monohydrate, before hypophysectomy. This was performed on diabetic rats by the parapharyngeal route. Completeness of pituitary removal was confirmed at necropsy.

Two diets were employed, a stock diet and one containing 60% glucose. The stock diet had the following composition: 67% ground whole wheat; 15% casein; 7.5% powdered skim milk; 0.75% NaCl; 1.5% $CaCO_3$; 6.7% lard; and 1% of a fish oil con-

Received October 17, 1956.

[1] This work was supported by a contract from the United States Atomic Energy Commission and by a grant from Eli Lilly and Company.

taining 3000 U.S.P. units of vitamin A and 400 international chick units of vitamin D. To this mixture was also added a few drops of potassium iodide. This stock diet is estimated to contain 57% carbohydrate.

The other diet had the following composition: 60% glucose; 22% casein; 9.8% celluflour; 0.2% of a vitamin B mixture; 2.0% liver Viobin; 6.0% of a salt mixture (9). Vitamins A and D in the form of a fish oil were fed every other day.

The rats had access to food until they were sacrificed, and at that time food was present in the stomachs of all rats.

The preparation of liver slices, their incubation with acetate-1-C^{14}, and the methods employed for determination of C^{14} in respired CO_2 and fatty acids have been described elsewhere (1).

Blood sugar was determined by the method of Mendel and Hoogland (10). Liver glycogen was determined by the method of Kemp *et al.* (11) after isolation according to Sjögren *et al.* (12).

RESULTS

Two experiments were carried out. In each, we compared the incorporation of the C^{14} of the incubated acetate-1-C^{14} to fatty acids and CO_2 by liver slices prepared from normal, diabetic, and diabetic-hypophysectomized rats. The rats of the first experiment were fed the stock diet; those of the second experiment received the 60% glucose diet. The results are recorded in Tables 1 and 2 and Figure 1.

Rats Fed the Stock Diet.—The food intake of the three groups of rats—normal, diabetic, and diabetic-hypophysectomized—is shown in Table 1 and Figure 1. The diabetic rats ingested about twice the amounts eaten

TABLE 1. HEPATIC LIPOGENESIS IN NORMAL, DIABETIC, AND DIABETIC-HYPOPHYSECTOMIZED RATS FED THE STOCK DIET

Slices were prepared from each liver, and duplicate 500±5 mg. portions were incubated for 3 hours at 37.5° in 5 ml. of a Krebs-bicarbonate buffer containing 1–2 μM of labeled acetate. Average values for each liver are recorded below.

Rat		Weight, gm.	Food intake* gm.	Blood glucose mg. per 100 ml.	Liver glycogen, per cent wet weight	Per cent of C^{14} of acetate-1-C^{14} recovered	
No.	Condition					As CO_2	As fatty acids
1	Normal	226	13		7.2	31.1	6.7
2		210	13		5.3	26.5	5.8
3		252	11		4.8	23.9	9.0
4		264	12		4.3	25.6	7.5
5	Diabetic	130	30	226	0.9	39.4	3.4
6		206	26	386	1.4	37.5	3.3
7		190	26	513	1.2	36.2	1.0
8		216	21	444	0.9	32.4	2.7
9		170	—	340	2.1	30.4	0.8
10	Diabetic hyped.†	214	10	368	—	38.4	2.3
11		212	13	168	0.9	41.4	1.5
12		170	10	200	1.3	32.4	0.8
13		170	15	247	—	33.8	1.0
14		192	20	364	2.7	32.5	3.5

* Food intake was measured daily. The values given are the averages of the last two days.
† Diabetic-hypophysectomized. Rats 10, 11, 13, 14 were sacrificed 14 days after hypophysectomy. Rat 12 was sacrificed 70 days after hypophysectomy.

by the normal rats. It is of interest to note, however, that the daily food intake of the Houssay rat is in the normal range. The blood glucose values of the Houssay rats were high, and the range was similar to that observed in the diabetic rats.

No marked difference in the $C^{14}O_2$ recoveries was observed in the three groups of rats studied. Less of the added C^{14} was incorporated into fatty acids by the liver slices prepared from the diabetic rats, and removal of the pituitary glands of diabetic rats failed to increase hepatic lipogenesis above the level observed in the diabetic group.

Rats Fed the 60% Glucose Diet.—It is again evident, from the data presented in Table 2, that the food intake of the diabetic rat is far greater than that of the normal rat, and that the food intake of the diabetic-hypophysectomized rat is about in the normal range.

It should be noted that the values for C^{14}-fatty acid recoveries were higher for the livers of the rats fed the 60% glucose diet (Table 2) than for the livers of rats fed the stock diet (Table 1).

Hepatic lipogenesis, as judged by the incorporation of C^{14} into fatty

TABLE 2. HEPATIC LIPOGENESIS IN NORMAL, DIABETIC, AND DIABETIC-HYPOPHYSECTOMIZED RATS FED THE 60 PER CENT GLUCOSE DIET

Duplicate 500 ±5 mg. portions of slices prepared from each liver were incubated as described in Table 1.

Rat		Weight, gm.	Days 60% glucose diet fed	Food intake,* gm	Blood glucose, mg. per 100 ml.	Liver glycogen, per cent wet weight	Per cent of C^{14} of acetate-1-C^{14} recovered	
No.	Condition						As CO_2	As fatty acids
1	Normal	196	3	12		5.1	14.8	15.8
2		154	3	12		4.4	11.0	36.5
3		205	7	11		8.8	37.7	29.3
4		182	7	12		6.0	29.1	35.0
5		195	24	10		3.6	27.2	31.2
6		206	24	10		—	29.1	12.4
7		198	10	13		6.3	45.8	15.3
8		210	10	13		4.7	45.6	16.6
9	Diabetic	214	10	21	468	1.1	38.1	2.9
10		152	10	20	615	4.9	36.2	3.7
11		162	10	24	474	4.0	39.4	10.6
12		126	10	29	581	1.4	38.5	2.4
13		158	10	30	224	—	57.1	4.7
14		168	10	25	294	—	55.4	3.4
15		178	10	20	464	4.5	24.0	3.7
16	Diabetic-hyped.†	162	10	9	451	1.7	30.3	14.5
17		152	10	11	536	1.5	32.6	14.7
18		151	10	10	432	1.5	33.0	15.6
19		165	15	12	635	—	20.5	23.9
20		168	15	15	620	3.0	18.8	12.2
21		145	10	13	212	—	56.0	6.7
22		184	10	16	520	1.2	52.8	9.2
23		182	10	19	—	1.8	47.6	6.1

* Food intake was measured daily. The values given are the averages of the last two days.
† Diabetic-hypophysectomized. These rats were sacrificed 15–22 days after hypophysectomy.

acids, was lower in the diabetic than in the normal; the average values for normal and diabetic livers were 24 and 4.5%, respectively. The fatty acid-C^{14} recoveries observed with the Houssay rats ranged from 6.1 to 24% (average 13); and only one of the eight values was above 16. These values for the livers of the Houssay rats, although above the average value for the livers of diabetic rats, are nonetheless significantly lower than the average observed with the normal livers.

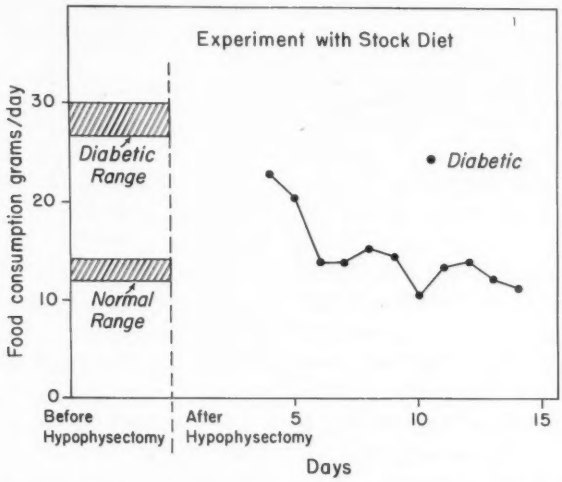

FIG. 1. The daily food consumption (stock diet) of alloxan-diabetic rats before and after hypophysectomy. Each point represents the average of five values.

COMMENT

The importance of available carbohydrate to hepatic lipogenesis in the hypophysectomized rat has been dealt with elsewhere (1). It was shown that, provided the hypophysectomized rat is fed a diet very high in available carbohydrate, hepatic lipogenesis proceeds at normal rates. A comparison of the corresponding values in Tables 1 and 2 can leave no doubt that the availability of carbohydrate is also of importance in the case of the Houssay rat.

The significant finding noted here is that *hypophysectomy fails to restore to normal the capacity of the diabetic liver to convert acetate carbon to fatty acids.* In the case of the diabetic rat fed the stock diet, hypophysectomy had no effect upon the extent of hepatic lipogenesis. Hypophysectomy did increase the extent of hepatic lipogenesis in the liver of the diabetic rat fed the 60% glucose diet, but the values observed for the conversion of acetate carbon to fatty acids by the livers of the diabetic-hypophysectomized rats were about half those obtained with normal rats. These findings in the rat are not in complete agreement with those observed in the

cat by Brady, Lukens, and Gurin (2). According to these investigators, fatty acid synthesis in the liver of the Houssay cat is normal.

Because the conversion of acetate carbon to fatty acids proceeds at normal rates in the livers of *hypophysectomized rats fed a high glucose diet* (1), we concluded that the maintenance of normal hepatic lipogenesis does not require anterior pituitary hormones. Thus, our failure to find normal hepatic lipogenesis in the Houssay rat fed the same high glucose diet serves to emphasize again the importance of insulin for hepatic lipogenesis.

SUMMARY

Normal, alloxan-diabetic, and hypophysectomized-diabetic (Houssay) rats were fed either a stock diet, in which whole wheat was the principal carbohydrate source, or a synthetic mixture containing 60% glucose. Daily measurements of food intake showed that the diabetic rats ingested about twice as much food as did the normal rats, and that the range of food intake of the Houssay rat was about the same as that of the normal rat.

Slices were prepared from the livers of these three groups of rats, and their capacity to convert the C^{14} of acetate-1-C^{14} to fatty acids was determined. Lipogenesis was depressed in the liver of the diabetic rat. Hypophysectomy failed to increase the extent of lipogenesis in the liver of the diabetic rat fed the stock diet. Hypophysectomy did increase lipogenesis in the livers of diabetic rats fed the 60% glucose diet, but *in these Houssay rats the extent of lipogenesis was still below normal.*

The significance of anterior pituitary hormones and of insulin in hepatic lipogenesis in the rat is discussed.

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THE EFFECTS OF ETHINYLANDROSTENEDIOL 3-CYCLOHEXYLPROPIONATE ON THE REPRODUCTIVE TRACT OF MALE AND FEMALE RATS

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A RECENT report from this laboratory (1) indicated that the biological activity of 17α-ethinyl-Δ^5-androstene-3β,17β-diol was predominantly estrogenic in nature and that esterification of the 3-hydroxyl group resulted in a change of the ratio of uterine growth stimulating (UGSt) to testicular growth suppressing (TGSp) activities. The smaller molecular weight esters (1 to 4 carbons) were considerably more estrogenic than the parent alcohol whereas the ability to suppress the growth of immature rat testes was affected only slightly. In contrast, the UGSt potency of several of the high molecular weight esters was greatly reduced without proportionate decrease in testicular growth suppressing activities.

This report presents data from a variety of experiments designed to establish the activity profile of one of the high molecular weight esters, 17α-ethinyl-Δ^5-androstene-3β,17β-diol 3-cyclohexylpropionate (hereafter referred to as ethandrostate), with special reference to its effects on the reproductive system of the male and female rat. The relative estrogenic, androgenic and gonad suppressing activities of this steroid are described and appropriate comparisons have been made with the behavior of several chemically different phenolic estrogens in terms of relative estrogenicity and pituitary inhibition as manifested by changes induced in the gonads and sex accessory organs.

MATERIALS AND METHODS

Male and female rats of the Sprague-Dawley strain were used. These animals were housed in temperature regulated and air-conditioned quarters. Laboratory chow and water were provided *ad libitum*. The test materials were administered subcutaneously as oil solutions or as aqueous suspensions. Cottonseed oil to which ethanol was added to 10% v/v served as oil vehicle. Aqueous suspensions were prepared by triturating the crystalline material in mortar and pestle with gradual addition of water to which one drop of Tween 80 per 5 ml. of water had been added as a dispersing agent. The amount of test substance to be injected was always contained in 0.2 ml. of vehicle.

Assays of estrogenic activity. Estrogenicity was assessed on the basis of stimulation of growth of the uterus of immature female rats and of induced cornification of vaginae of castrate female rats. The uterine growth tests were conducted in accordance with the procedures outlined by Lauson *et al.* (2). The material to be tested was administered subcutaneously once a day for 3 days to female rats weighing 35 to 40 gm. Twenty-four

Received October 17, 1956.

hours after the last injection the uteri were removed, blotted and weighed on a micro-torsion balance. The dose of each compound capable of producing 50% of maximum uterine growth (ED_{50}) was calculated from log-probit graph paper by the method of Miller and Tainter (3). The maximum uterine weight used for ED_{50} calculations was 110 mg., obtained from a previously constructed estrone (USP) standard dose-response curve. A modified Allen-Doisy (4) test was used to assay the ability of the test agents to induce vaginal cornification of castrate rats. The material for assay was injected at 2:00 P.M. on day one and at 10:00 A.M. on day two of the assay period. Vaginal smears were taken and read at 4:00 P.M. on day two and at 10:00 A.M. on days three and four. Smears showing predominantly nucleated or cornified epithelial cells were graded as positive.

Assay of androgenic activity. The degree of androgenicity of the test agents was assayed using weight response of sex accessory organs of castrate male rats as the index. Rats weighing 125–150 gm. at operation were brought to experiment on the seventh post-castration day. Injections were given once a day for five days. On the eighth day of the assay, and 72 hours after the last injection, the animals were sacrificed under chloroform. The seminal vesicles and ventral prostate glands were removed, cleaned, blotted between filter papers and weighed on a micro-torsion balance.

Methods for assessing pituitary-gonadal effects: The effects of ethandrostate on the pituitary-gonadal system were determined by means of the following tests: 1) *Testicular growth experiments.* Modification of rate of growth of testes of immature rats (35 to 40 gm.) was used as a gauge of altered pituitary function. The test substance was administered once a day for 5 days. On the third day after the last injection the testes were removed, cleaned, weighed and compared with weight of testes from comparable untreated controls. 2) *Growth of the sex accessory organs of young adult rats.* Male intact rats weighing 170 to 190 gm. were given the test agent once daily 6 days a week for 2 weeks. Rats which were castrated on the first day of the test as well as intact untreated rats were used as controls. Within 24 hours after the last injection, the rats were sacrificed and the testes, seminal vesicles, ventral prostate and pituitary were removed, cleaned and weighed. In tests designed to measure duration of activity, the test material was administered subcutaneously as a single injection. A group of 5 to 6 rats was sacrificed on post-injection day 7, 14, 21, 28, 35 and 42 and examined as described above. 3) *Ovarian changes in reproductively mature female rats.* Ethandrostate was administered daily except Sunday for 2 weeks to intact female rats weighing 180 to 200 gm. At autopsy the ovaries, uterus, adrenals and pituitary were removed, cleaned and weighed. The intraluminal fluid was expressed before weighing the uteri. Intact females which received only the vehicle served as controls. 4) *Disruptance of pregnancy.* Aqueous suspensions of ethandrostate were injected subcutaneously into mated female rats on the day that sperm were observed in the vaginal smears (day one of pregnancy) and continued daily six days a week for two weeks. On the 15th day of pregnancy, 24 hours after the last injection, confirmation of pregnancy was obtained by inspection of the uteri of treated and control animals. The embryonic masses were counted and measured transversely with calipers *in situ.* Embryos were judged to be viable if they were irritable to probing or if the pumping action of the heart could be seen.

RESULTS

1. *Estrogenic activity.* The growth promoting effect of ethandrostate on the uterus of immature rats is shown by the data in Table 1. The effects of estrone and estradiol-17β under the same conditions are included for comparison. The dose which produced 50% of maximum uterine stimulation (ED_{50}) was determined for each substance from log-probit graph

TABLE 1. THE ESTROGENIC ACTIVITY OF ETHANDROSTATE COMPARED WITH PHENOLIC ESTROGENS BASED ON GROWTH RESPONSE OF IMMATURE RAT UTERUS

Compound	Dose, mg./kg./day	No. of rats	Uterine weight, mean ± s.e., mg.	Per cent of maximum stimulation[1]	Approx. ED_{50} ± s.e.,[2] mg./kg./day
Estrone (C)	0	5	24.6 ± 2.2	0	
	.0025	5	31.8 ± 2.3	8	
	.005	5	49.0 ± 7.2	28	
	.010	5	70.5 ± 12.5	54	.009 ± .003
	.020	5	75.2 ± 2.4	60	
	.040	5	110 ± 5.1	100	
	.080	5	102 ± 8.2	91	
Estradiol-17β (C)	0	8	38.4 ± 3.4	0	
	.00037	8	49.5 ± 3.5	15	
	.00075	8	77.8 ± 4.9	55	.0008 ± .0003
	.0015	8	89.8 ± 7.1	72	
	.0030	8	96.8 ± 4.2	82	
Ethandrostate (C) (aqueous susp.)	0	6	28.7 ± 2.1	0	
	2.5	5	43.4 ± 2.1	18	
	5.0	6	54.9 ± 3.7	32	9.0 ± 4.5
	10.0	6	71.9 ± 3.3	53	
	20.0	6	89.7 ± 3.8	75	
Ethandrostate (C) (oil solution)	0	6	23.6 ± 1.2	0	
	10	6	51.4 ± 5.7	32	
	20	6	63.0 ± 4.7	45	22.0 ± 7.8
	40	6	84.2 ± 5.5	70	

[1] Calculated as per cent increment in uterine weight over control value with 110 mg. representing maximum (100%) uterine response.

[2] Calculated according to Miller and Tainter (1944). Approximate s.e. $ED_{50} = 2s/\sqrt{2N}$ where $2s$ is the estimated increment in dosage necessary to increase the effects by 2 probits.

(C) Control groups.

paper by the method of Miller and Tainter (3) and the relative potency was appraised by establishing the ratio of the ED_{50} doses. Ethandrostate administered in oil solution was 1/27,500th as active as estradiol-17β and 1/2440th as active as estrone in promoting uterine growth. Aqueous suspensions of ethandrostate exhibited approximately 2½ times more activity than did the solution in oil.

Ethandrostate was completely effective in changing the cellular characteristics of the castrate female rat vagina from the atrophic to the fully estrous type as shown by vaginal smears (Table 2). On a weight basis, the dosage of ethandrostate in oil which was required to produce positive vaginal smears in 50 per cent of the rats treated was approximately 35,000 times the equipotent dose of estradiol-17β in oil. Again the aqueous suspensions of ethandrostate were significantly more active (2½ times) than the oil solutions. This difference in activity could be due to differences in absorbability.

2. *Androgenic activity.* The fact that ethandrostate is an androstane derivative which is chemically not greatly different from steroids possessing considerable androgenicity, prompted studies to determine the androgenic activity of ethandrostate relative to testosterone propionate. The results

TABLE 2. ESTROGENIC ACTIVITY OF ETHANDROSTATE AS EVIDENCED BY INDUCED CORNIFICATION OF CASTRATE RAT VAGINA

Treatment	Dose, mg./kg./day	No. of rats	No. of rats showing positive responses	Per cent positive	Approx. $ED_{50} \pm$s.e.[1]
Estradiol-17. (C)	0.0	10	0	0	
	0.000125	10	4	40	
	0.00025	10	8	80	$0.00015 \pm .00002$
	0.0005	8	8	100	
Ethandrostate (C) (oil solution)	0.0	10	0	0	
	4.0	10	2	20	
	8.0	10	9	90	5.2 ± 0.6
	16.0	10	10	100	
Ethandrostate (C) (aqueous suspension)	0.0	18	0	0	
	1.0	18	0	0	
	2.0	19	7	37	2.1 ± 0.2
	4.0	11	11	100	

[1] Calculated by method of Miller and Tainter (1944) according to formula: approx. s.e. $ED_{50} = 2s/\sqrt{2N}$, where $2s$ is the estimated increment in dosage necessary to increase the effects by 2 probits.
(C) Control groups.

of the assay are presented in Table 3. Threshold responses of the sex accessory organs of castrated male rats were elicited by ethandrostate at a dose level of 80 mg./kg./day for 5 days and by testosterone propionate at 0.125 mg./kg./day. These minimal responses to ethandrostate do not permit an accurate estimate of relative potency, but do indicate that ethandrostate is very feebly androgenic at most. It should be mentioned that the degree of response obtained with ethandrostate, particularly with respect to the seminal vesicles, can be elicited with estrogens (*cf.* Table 5).

3. *Effect of Ethandrostate on the Gonads and Reproductive Structures of Intact Male and Female Rats.*

TABLE 3. THE ANDROGENIC ACTIVITY OF ETHANDROSTATE COMPARED WITH TESTOSTERONE PROPIONATE IN CASTRATE MALE RATS

Treatment	Dose mg./kg./day	No. of rats	Body weight, gm.		Sex accessory organ weights	
			Initial	Final	Seminal vesicle, mg. (mean $\pm$s.e.)	Ventral prostate, mg. (mean $\pm$s.e.)
Control	nil[1]	5	154	180	12.0 ± 0.95	10.5 ± 0.7
Testosterone propionate (in oil)	0.0625	5	148	177	13.6 ± 2.1	13.2 ± 1.5
	0.125	5	147	177	17.6 ± 1.3	18.5 ± 1.0
	0.25	5	153	179	27.0 ± 3.2	24.8 ± 2.4
	0.50	5	149	185	56.6 ± 5.2	45.6 ± 1.6
	1.00	5	156	193	92.4 ± 8.0	67.4 ± 2.9
Control	nil[1]	5	151	180	11.1 ± 0.5	10.2 ± 1.1
Ethandrostate (in oil)	10.0	5	160	181	13.3 ± 1.6	11.9 ± 0.7
	20.0	5	158	185	14.7 ± 1.5	15.5 ± 0.7
	40.0	5	156	175	15.2 ± 1.3	14.7 ± 1.1
	80.0	5	153	166	16.9 ± 1.3	14.8 ± 2.0

[1] Vehicle only.

TABLE 4. SUPPRESSIVE EFFECT OF ETHANDROSTATE ON THE GROWTH OF TESTES OF IMMATURE MALE RAT

Treatment	Dose, mg./kg./day	No. of anmals	Body weight		Testicular weight, mg. (mean ±s.e.)	Per cent Suppression of testicular growth[1]	Sex accessory organ weight	
			Initial	Final			Seminal vesicle mg.	Ventral prostate, mg.
Controls:								
Pretreatment	—	10	38	—	220 ±10	—	5.2	18.6
Posttreatment	—	9	42	66	544 ±22	—	8.5	33.2
Ethandrostate[2]	5.0	5	41	60	470 ±27	23	6.4	24.5
	7.5	5	37	70	327 ±55	67	7.0	22.5
	10.0	5	40	62	295 ±21	77	5.6	24.2
	12.5	5	38	71	277 ±30	82	6.7	24.1
	20.0	5	40	58	204 ±49	105	5.4	17.2

[1] Calculated on the basis of difference in growth of testes from treated versus untreated rats.
[2] Aqueous suspension.

a) *Testicular growth suppressing activity.* The ability of ethandrostate to suppress the growth of infantile rat testes was related to dose over a range of 5 to 20 mg./kg/day for 5 days (Table 4). The per cent suppression of growth was calculated on the basis of difference in growth of testes of treated compared with untreated rats. A highly significant ($P<.01$) suppression of testicular growth (67%) was produced by 7.5 mg./kg/.day and 20 mg./kg./day produced complete (105%) inhibition. These dose levels did not stimulate the seminal vesicle or prostate glands.

To determine whether the effect of ethandrostate on testicular growth was due to a direct or indirect effect, 10 I.U. of pregnant mare serum gonadotrophin (PMS) was administered daily for 5 days to 35 to 40 gm. rats which received 10 mg./kg./day of ethandrostate as an aqueous suspension concurrently. Rats given only the gonadotrophin, as well as un-

TABLE 5. COMPARISON OF ETHANDROSTATE WITH PHENOLIC ESTROGENS WITH RESPECT TO NUMBER OF ESTROGENIC UNITS OF EACH REQUIRED FOR TESTICULAR GROWTH SUPPRESSION

Compound	*A* Dose required to effect 50% suppression of testicular growth	*B* Dose required to effect 100% suppression of testicular growth	*C* Calculated dose equivalent to one estrogenic unit[1]	Dose ratio	
				A:C	B:C
	mg./kg./day	mg./kg./day	mg./kg./day		
Estradiol-17β	0.041	0.288	0.0008 ± .0003[2]	51:1	360:1
Estrone	0.068	0.477	0.009 ± .003	7.6:1	53:1
Diethylstilbestrol dipalmitate	0.107	0.499	0.037 ± .013	2.9:1	13.5:1
Ethandrostate (aqueous suspension)	7.2	15.3	9.0 ±4.5	0.8:1	1.6:1

[1] One estrogenic unit equals one uterine growth stimulating ED_{50}.
[2] Approximate ED_{50} ±s.e.

treated rats, served as controls. The results of this test are presented graphically in Figure 1. The testicular growth suppressing effect of ethandrostate was completely obliterated by concurrent administration of PMS. On the other hand the ability of the testis to produce androgen in response to gonadotrophin stimulation, as judged by the spurt in seminal vesicle growth exhibited in the dually treated rats, was not disturbed by ethandrostate. The evidence indicates that the testicular growth suppressing action of ethandrostate is indirect and presumably is due to inhibition of

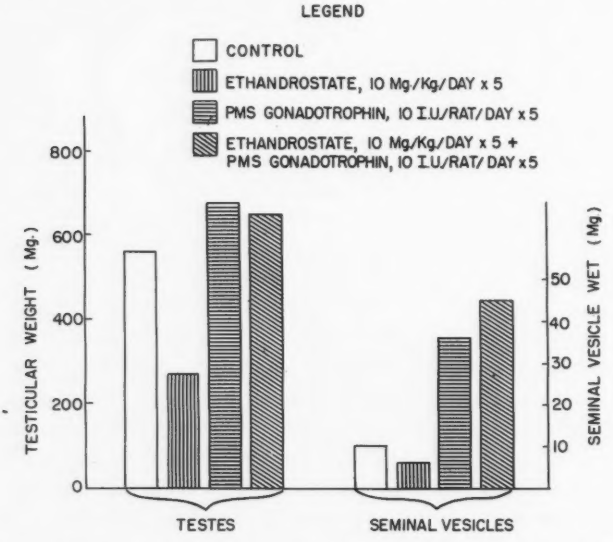

FIG. 1. Neutralization of the gonad suppressing effects of ethandrostate by concurrent administration of gonadotrophin (PMS)

the pituitary output of gonadotrophin, a well known activity manifested by both estrogenic and androgenic substances (*cf.*, 5).

The testicular growth-arresting activity of ethandrostate has been compared with that of three well known phenolic estrogens, estradiol-17β, estrone and diethylstilbestrol dipalmitate, by plotting the degree of suppressive effect in terms of estrogenic equivalents (Figure 2). The uterine growth stimulating ED_{50} for each agent is taken as one estrogenic unit. It is immediately apparent from Figure 2 that these estrogens differ markedly with respect to the number of estrogenic units (UGSt ED_{50}'s) of each required to produce suppression of testicular growth. These differences are shown even more clearly in Table 5, which presents for each compound the estrogenic potency needed to bring about 50 and 100% suppression of testicular growth as determined from the appropriate points on the activity curves in Figure 2. A comparison of dose ratios indicates that diethylstilbestrol dipalmitate, estrone and estradiol required 4, 10 and 60 times

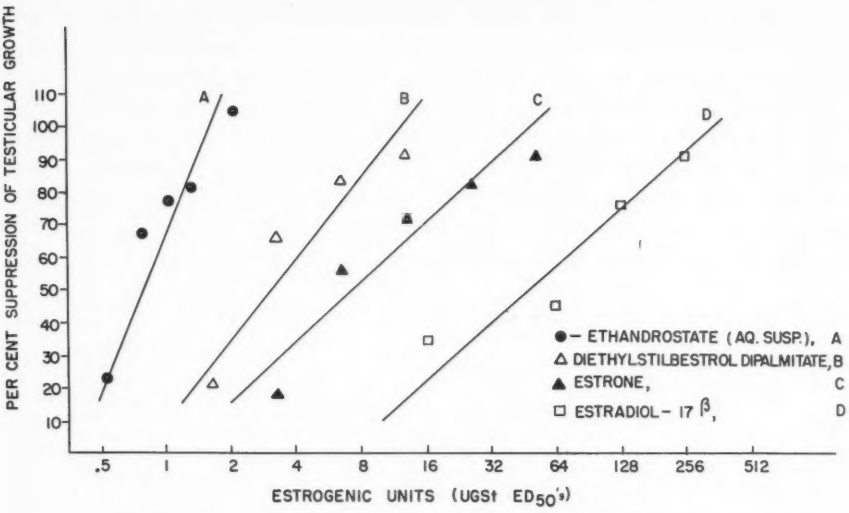

FIG. 2. Estrogenic requirements of various estrogens for attainment of testicular growth suppression of immature rats.

more estrogenic units than did ethandrostate to produce 50% suppression of testicular growth. To produce 100% suppression, nearly 8, 30 and 225 times more estrogenic units of diethylstilbestrol dipalmitate, estrone and estradiol were needed than were required of the non-phenolic ethandrostate. It is apparent from these data that ethandrostate produced more suppression of testicular growth per unit of intrinsic estrogenic activity than did any of the representative estrogens studied.

b) *Effect of ethandrostate on the reproductive system of intact mature male rats.* Ethandrostate administered subcutaneously to mature male rats brought about atrophy of the seminal vesicle and ventral prostate glands which in degree was proportional to dose within the range of 2 to 20 mg./kg./day for two weeks (Table 6). Aqueous suspensions of this steroid were considerably more active than the oil solutions, which might be ex-

TABLE 6. THE EFFECT OF ETHANDROSTATE ON THE REPRODUCTIVE SYSTEM OF INTACT MALE RATS

Treatment	No. of animals	Dose, mg./kg.	Body weight			Organ weights (mean ± s.e.)			
			Initial	Final	Δ gm.	Pituitary	Testes	Seminal vesicle	Ventral prostate
						mg.	mg.	mg.	mg.
Castrate Control	10	—	176	223	+47	10.1±0.4	—	42± 4	21±0.9
Intact Control	10	0	176	250	+74	7.6±0.3	2805± 59	223±10	231±21
Ethandrostate (oil solution)	5	2	176	248	+72	7.8±0.3	2780± 97	187±23	164±16
	5	8	180	235	+55	8.4±0.3	2730± 50	115± 6	121± 7
	5	16	186	216	+30	10.2±0.3	2510± 60	89±20	106±14
	4	20	183	209	+26	10.5±0.5	2260±220	66± 8	94± 9
Intact Controls	6	0	191	256	+65	8.8±0.4	2890± 77	190±10	202±27
Ethandrostate (aqueous suspension)	7	2	188	258	+70	8.5±0.3	2870± 60	178±10	180±17
	7	4	190	256	+66	9.7±0.3	3040± 51	142±11	169±11
	6	8	191	231	+40	9.8±0.3	2570± 72	63± 7	76± 9
	6	16	188	216	+28	10.7±0.6	2580± 59	56±10	52±12

pected on the basis of the greater estrogenic potency exhibited by the suspension (*cf.* Tables 1, 2). The maximum degree of sex accessory organ atrophy induced by ethandrostate was not greatly different from that observed two weeks following the removal of the testes from rats of similar age and weight. It should be noted that this steroid exerted a retarding effect on total body growth; however, the degree of sex accessory organ atrophy was disproportionately greater than the general growth retarding effects. On the other hand, the changes in testicular weight as shown in Table 6 were similar in degree to the body weight changes so that the

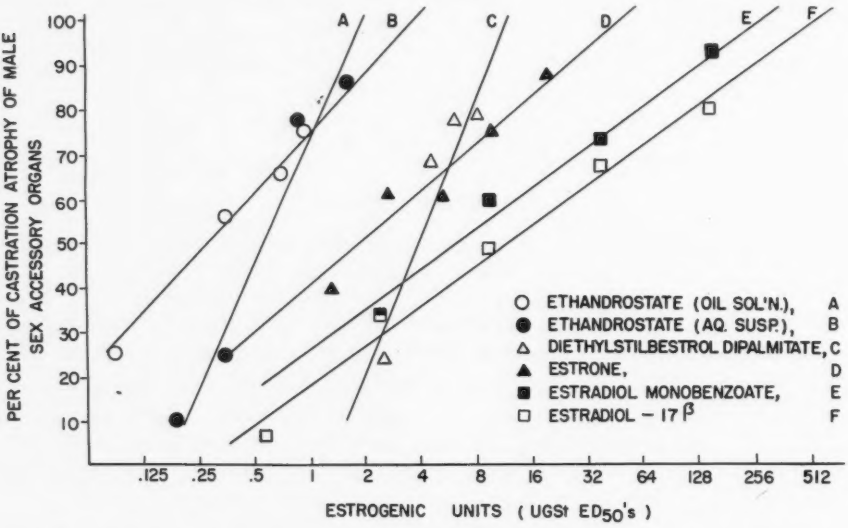

FIG. 3. Estrogenic requirements of various estrogens for attainment of atrophy of male rat sex accessory organs.

influence of ethandrostate on testicular mass within the two week period of this test was questionable. A moderate but statistically significant ($P < .02$) increase in pituitary mass was observed at the high dose levels of ethandrostate.

Various highly active phenolic estrogens were compared with ethandrostate with respect to the number of estrogenic units (UGSt ED_{50}'s) of each required to produce atrophy of the sex accessory organs of mature male rats. Activity curves correlating these two parameters are shown in Figure 3. Differences in slope as well as position of the curves on the estrogenic unitage log-scale are apparent for estradiol-17β, estradiol monobenzoate and estrone on the one hand, and for diethylstilbestrol dipalmitate and ethandrostate on the other. These differences are emphasized by the data in Table 7 which shows the number of ED_{50} estrogenic units of each of the various estrogens needed to bring about 50 and 100%

TABLE 7. COMPARISON OF ETHANDROSTATE WITH PHENOLIC ESTROGENS WITH RESPECT TO NUMBER OF ESTROGENIC UNITS OF EACH REQUIRED TO EFFECT 50 AND 100 PER CENT OF CASTRATION ATROPHY OF SEX ACCESSORY ORGANS OF MATURE MALE RAT

Compound	*A* Dose required[1] to effect 50% of castration atrophy	*B* Dose required[1] to effect 100% of castration atrophy	*C* Calculated dose equivalent to one estrogenic unit[2]	Dose ratio A:C	Dose ratio B:C
	mg./kg./day	mg./kg./day	mg./kg./day		
Estradiol-17β	0.010	0.409	0.0008 ± .0003[3]	13:1	512:1
Estradiol-monobenzoate	0.007	0.358	0.001 ± .00025	7:1	358:1
Estrone	0.019	0.522	0.009 ± .003	2:1	58:1
Diethylstilbestrol dipalmitate	0.148	0.481	0.037 ± .013	4:1	13:1
Ethandrostate:					
a) aqueous suspension	5.4	18.0	9.0 ± 4.5	0.6:1	2:1
b) oil solution	6.6	88.0	22.0 ± 7.8	0.3:1	4:1

[1] Calculated from Fig. 3.
[2] One estrogenic unit equals one uterine growth stimulating ED_{50}.
[3] Approximate ED_{50} ± s.e.

of castration atrophy of the ventral prostate and seminal vesicles. The values shown in Table 7 were derived from the activity curves in Figure 3. Ethandrostate, either in oil solution or as an aqueous suspension, effected the greatest degree of atrophy of the sex accessory organs per unit of estrogenicity (UGSt ED_{50}), whereas estradiol and its monobenzoate produced the least change per estrogenic unit. Estrone and diethylstilbestrol dipalmitate occupied a rank intermediate between ethandrostate and estradiol. The dose ratios as shown in Table 7 are similar, for all practical purposes, to those obtained when testicular growth suppression and estrogenicity were the selected parameters. It should be pointed out here that the observed differences in ratios of pituitary inhibitory dose to estrogenic dose for these estrogens appear to reflect the variations in degree of estrogenicity rather than differences in pituitary inhibitory activity.

The observation that ethandrostate and diethylstilbestrol dipalmitate, which are characterized by highly lipophilic ester groups, manifested the lowest dose ratios of pituitary inhibitory to estrogenic activity, suggested that the variation in dose ratios exhibited by the various estrogens might be merely a reflection of differences in rates of absorption, distribution, degradation and/or removal of these substances. Because the manifestations of biological activity of an exogenous agent are largely functions of duration of exposure and concentration relative to sensitivity of the target organ, the functional role of the lipophilic ester groups was examined by comparing ethandrostate and its parent alcohol, ethinylandrostenediol, with estradiol-17β on the basis of *duration* of the pituitary inhibition induced by single doses. For these studies 100 μg. of estradiol, 25 mg. of ethinylandrostenediol and 38 mg. of ethandrostate (equivalent to 25 mg.

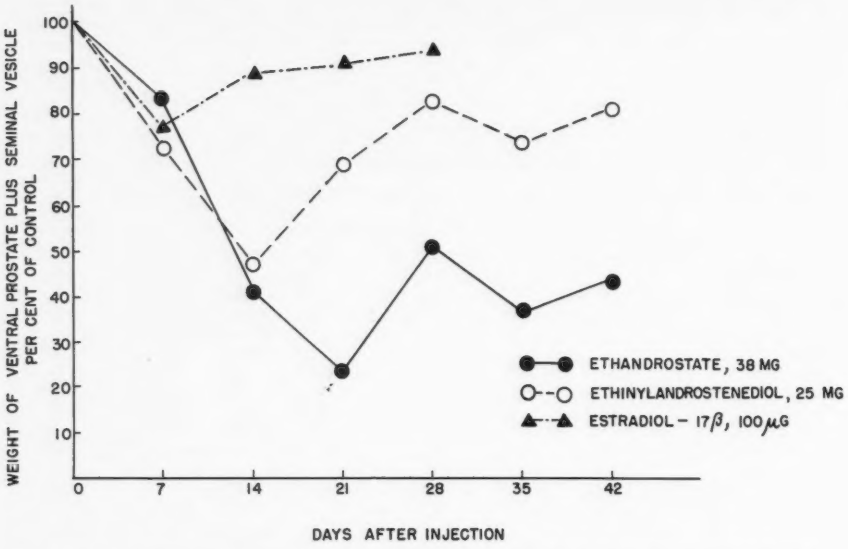

FIG. 4. Duration of sex accessory organ atrophy of mature male rats administered a single dose of ethandrostate, ethinylandrostenediol or estradiol.

of the free alcohol) were administered as single injections to young mature male rats, weighing 170–190 gm. These doses were 720, 30 and 24 times the uterine growth stimulating ED_{50} for estradiol, ethinylandrostenediol and ethandrostate, respectively. The duration of activity is indicated by the curves in Figure 4 which show the weekly changes of the composite weight of the ventral prostate and seminal vesicles of treated rats expressed as a per cent of that of control rats. It is apparent from inspection of the curves that ethandrostate exhibited a considerably greater duration of pituitary inhibition than did either the parent alcohol or estradiol. Six weeks after the injection of ethandrostate the weights of the sex accessory organs were still only 43% of those of respective control organs. The free alcohol, ethinylandrostenediol, exhibited a sustained activity which was also definitely greater than that of estradiol. The duration of activity of these three steroids was inversely proportional to the equivalent number of uterine growth stimulating ED_{50}'s of each which was administered. These seemingly paradoxical findings strongly suggest that differences in rates

TABLE 8. THE EFFECT OF ETHANDROSTATE ON THE REPRODUCTIVE SYSTEM OF INTACT MATURE FEMALE RATS

Treatment	Dose, mg./kg. /day	No. of animals	Body weight, gm.			Organ weights, mg. (Mean and s.e.)			
			Initial	Final	Δ	Ovaries	Uterus	Pituitary	Adrenal
None	—	5	180	198	+18	63.5±3.9	350±34	11.6±0.65	52.7±2.4
Ethandrostate	2	5	184	194	+10	51.1±2.1	382±20	10.2±0.75	53.8±4.8
Ethandrostate	4	5	185	198	+13	42.6±2.3	383±11	11.7±0.80	58.2±2.3
Ethandrostate	8	5	182	186	+ 4	32.3±2.3	486±37	13.2±0.93	52.7±4.3

of absorption and/or degradation of the various estrogens might contribute significantly to variation in dose ratios of pituitary inhibitory to estrogenic activity.

c. *Effect of ethandrostate on reproductive tract of intact mature female rats.* Ethandrostate administered to intact mature female rats brought about marked regressive changes in the ovaries (Table 8). A statistically significant reduction ($P = .02$) in the mass of the ovaries was observed at a dose level of this steroid equivalent to 2 mg./kg./day for 2 weeks. Larger doses effected correspondingly greater degrees of ovarian regression. Grossly the atrophied ovaries appeared pale and were noticeably deficient in luteal tissue. Many small follicles could be seen near the surface. The general impression was that these small ovaries contained more follicles than pos-

TABLE 9. ANTI-GESTATIONAL EFFECT OF ETHANDROSTATE

Treatment	Dose, mg./kg./day	No. of rats	No. of rats pregnant on 15th day	Average no. of embryonic masses per rat	Average no. of viable embryos per rat	Mean width of uterus at site of embryonic masses
						mm. and range
Control	—	4	4	8.5	8.0	11.9 (10.9–12.6)
Ethandrostate (aqueous susp.)	5	5	5	8.4	0	6.1 (4.2–10.0)
	20	6	1	0.67	0	1.1 (0– 6.5)

sessed by ovaries from the untreated controls. Only the high dose of 8 mg./kg./day produced a significant uterine stimulation. No real changes in the weight of the pituitaries or adrenals were observed even with the high dose, which exhibited a suppressive effect on body growth.

d. *Disruptance of pregnancy.* The data in Table 9 show that although 5 mg./kg./day of ethandrostate during first two weeks of pregnancy did not interfere with implantation, it did exert a definite abortifacient effect in that the embryonic masses were considerably smaller than normal and none of the embryos showed signs of life. The higher dose of 20 mg./kg./day prevented implantation in 5 of the 6 rats treated. The ovaries from these rats contained corpora lutea which were smaller than those observed in ovaries from 15-day pregnant control rats. The observed ovarian changes induced by ethandrostate in pregnant rats suggested an anti-luteinizing action of this steroid.

DISCUSSION

The accumulated data indicate that the activity of ethandrostate is predominantly estrogenic. Androgenic activity can be demonstrated for this steroid only at dose levels which are many times those required to

stimulate the growth of the infantile rat uterus or to induce cornification of the castrate rat vagina. The unequivocal pituitary inhibitory activity observed for ethandrostate in tests wherein male and female rats were used is assumed to be a function of its estrogenicity.

From the studies reported here as well as from a previous report from this laboratory (1) the following structural modifications of estradiol-17β were observed to lower the dose ratio of pituitary inhibitory to estrogenic activity: 1) the presence of a C_{17}-ketone group as in estrone 2) C_{17}-ethinylation of estradiol as for ethinylestradiol, 3) saturation of ring A as in androstanediol and Δ^5-androstenediol, 4) ethinylation of the fully or partially saturated androstane derivatives, ethinylandrostanediol and ethinyl-Δ^5-androstenediol and 5) esterification with high molecular weight lipophilic acids such as palmitic and cyclohexylpropionic acids. It is conceivable that a particular chemical change might alter the pituitary inhibitory to estrogenic dose ratio of an estrogen by modifying its rate of absorption, rate of destruction or excretion, affinity for the target or responsive tissue cells or any combination of the three. It is inferred from differences in duration of action of various estrogens that absorption and degradation rates are important contributory factors to altered activity dose ratios.

It is noteworthy that significant suppression of testicular growth or decrease in weight of the sex accessory organs was not produced in rats with less than the equivalent of one uterine growth stimulating ED_{50} of the various phenolic estrogens. Although the experimental procedures in terms of strain, age and weight of animals as well as duration of treatment have varied from laboratory to laboratory, our observations are in general agreement with the mass of data reported for experiments of similar nature (6, 7, 8, 9). Our data also support the unit mechanism theory of regulation of pituitary gonadal functions advanced by Greep and Jones (10) with respect to the effect of estrogens on the pituitary production of FSH and LH (ICSH).

Ethandrostate appears to be a unique steroid in that it is a "non-phenolic estrogen" and in comparison with the phenolic estrogens possesses what appears from the data to be a relatively high degree of specificity as an inhibitor of pituitary gonadotrophic function.

The evidence suggests that a clinically useful degree of pituitary inhibition, with a minimum of undesired estrogenic manifestations, might be attained by establishment of optimum dosage and frequency of administration for the management of subacute endocrine imbalance.

SUMMARY

Ethandrostate (17α-Ethinyl-Δ^5-androstene-3β,17β-diol, 3-cyclohexylpropionate) was observed to be fully estrogenic at dose levels which were considerably lower than the androgenic threshold dose.

This non-phenolic steroid exhibited marked inhibitory influence on

pituitary gonadotrophin output as judged by its ability to suppress the growth of the testes of immature rats, to induce ovarian regression in young mature female rats, to bring about near castration level atrophy of the sex accessory organs of young mature males and to prevent or to disrupt pregnancy.

A comparison of ethandrostate with estradiol-17β, estrone, estradiol benzoate, and diethylstilbestrol dipalmitate was made in respect to the number of estrogenic units (uterine growth stimulating ED_{50}) of each required to produce marked pituitary inhibition. Ethandrostate was observed to effect the greatest degree of pituitary inhibition per unit of estrogenicity within the series of estrogens studied. The pituitary inhibitory action of ethandrostate is assumed to be a function of its estrogenicity despite the disparity in dose-effect ratio as compared with the dose ratios derived for other estrogens.

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DIFFERENCES IN VAGINAL SENSITIVITY OF MICE OF INBRED STRAINS; LOCALIZATION IN THE VAGINA[1]

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THE vaginal mucosa of mice of different inbred strains differs in responsiveness to exogenous estrogen (1, 2, 3, 4). More than four times as much estrogen was required to induce cornification in 50% ±10% of the vaginas of mice of some strains than was required by mice of other strains (4, 5). The differences in vaginal sensitivity were not correlated with susceptibility to mammary cancer (1, 3, 4, 6).

The strain differences in susceptibility of the vaginal mucosa may be due to the genetic constitution of mice of the different strains which result in (a) differences in the rate of inactivation or excretion of injected estrogen, (b) differences in the production of estrogen or substances that might antagonize or augment their action, or (c) differences in the responsiveness of the end organ.

The possibility that differences in inactivation or excretion of the hormones might occur was excluded largely when the topical application of the hormones to the vaginal mucosa revealed a similar order of the responses although with much smaller amounts of estrogen (5). The possibilities mentioned under items (b) and (c) above, however, could not be elucidated by such experiments.

Many tissues or glands of inbred mice have been transplanted from one mouse to another within the strain or into the first generation hybrids. The responsive tissues of mice of contrasting parental strains may be transplanted into their hybrid offspring. In this manner "genetically" different tissues can be followed in the same host and presumably under identical environmental conditions.

Strain differences in the capacity of the adrenal glands to form tumors in gonadectomized mice (7), of the lungs to form adenomas after the administration of carcinogens (8, 9), or of the testis to form interstitial cell tumors in estrogen-treated mice (10), have been studied using this technic. In all instances the strain specificity of the abnormal or neoplastic responses were determined by the responding organ rather than the environment. The possibility that the strain differences in vaginal sensitivity to

Received October 30, 1956.

[1] This investigation was supported in part by a grant from the National Cancer Institute, U.S.P.H.S., the Jane Coffin Childs Memorial Fund and the Anna Fuller Fund.

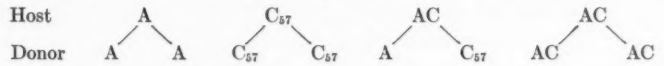
estrogens might be due to intrinsic differences in the end organ has been investigated (11). Quantitative strain specific responses of the vaginal mucosa are present in the end organ and not in the environment in which it is maintained, as shown by the studies reported below.

MATERIALS AND METHODS

The mice used were from 2 inbred strains (A and C_{57})[2] and reciprocal hybrids of these strains (AC). The two strains were selected because they had the most contrasting vaginal responses to estrogens (4).

The general plan of the experiment was as follows: *a*. The vaginas of young mice from 16 to 24 days of age (usually 16 days old), were transplanted subcutaneously into the lower lateral abdominal areas of young adult hosts (Figs. 1, 2). The external vaginal orifice of the donors was not open in mice at this age so that sterile grafts could be obtained. Also, the vaginal walls were thin but could be readily separated from the urethra and adjacent pelvic tissues. The dissections were done at low power magnification in opened sterile Petri dishes containing sterile, saline-dampened filter paper. Care was taken to exclude the cervical canal and lower uterine horns because preliminary experiences had shown that uterine secretions interfered with reading of the vaginal smears that were taken subsequently. *b*. One to two months later, after the transplants had become enlarged, they were opened and sutured to the adjacent skin to maintain the fistula. Some mice received 16.6 μg. of estradiol benzoate a week or more prior to this operation in order to increase the size of the grafts. *c*. Daily smears were taken of the exfoliated cells of the intact vagina and of the fistulated grafts. The smears were taken with a water dampened, flat tooth-pick and spread on a small drop of water on a slide. After drying the slides were stained with hematoxylin and eosin preparatory to microscopic examination. *d*. After the response of the intact vaginal mucosa and of the grafts to the intrinsic hormones had been followed for 2 to 22 cycles (Tables 4, 5) the mice were ovariectomized and the vaginal responses to injected estradiol benzoate[3] were determined during 4 to 27 tests on each animal. All injections were given subcutaneously. The hormone was dissolved in 0.05 ml. of sesame oil. The amounts of hormone administered ranged from 0.5 μg. to 0.03125 μg.

Four comblnations of experiments were designed so that the effects of transplantation as well as the effects of the different genetic potentialities of the tissues might be checked.



A few animals were discarded because one or both grafts prolapsed after being fistulated. These grafts had not become well fixed to the underlying muscle. A small number of grafts became infected during the course of repeated examinations. Observations were discontinued on the infected grafts. The mice were examined over periods ranging from 1 to 10 months.

The mice were maintained in groups of not more than 4 per cage ($12'' \times 6'' \times 6''$) with food (Purina Laboratory Chow) and water available at all times. The laboratory was air conditioned—72–74° C and $50 \pm 10\%$ relative humidity. Small groups of mice from the

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[2] These mice were originally derived from the colony maintained by Dr. L. C. Strong and have been maintained as a separate colony by brother-sister matings for 13 years.

[3] The estradiol benzoate was obtained in crystalline form and the solutions prepared by serial dilutions of a stock solution containing 1 mg. per cc. The hormone was generously supplied by Ciba Pharmaceutical Products, Incorporated.

4 groups were studied concurrently. Additional groups were added over a two-year period at irregular intervals.

All operations were done with sterile instruments but without rigid attempts to assure ascepsis. Sodium amytal and ether were the anesthetics used.

At the termination of the periods of experimentation the mice were killed and the transplanted vaginal tissues and intact genital tissues were removed, fixed, sectioned at 7 μ and stained with hematoxylin and triosin.

The vaginal smears were recorded at six stages; diestrus, indicated as (− −), early proestrus (− − +), late proestrus (− + +), estrus (+ +), early postestrus (+ + −), and late postestrus (+ − −). The typical estrous smears revealed cornified cells, singly or in small clumps and few, if any, nucleated cells. The late proestrous smears contained more than half cornified cells, the remainder being nucleated epithelial cells or "ghost" polymorphonuclear leukocytes. The early postestrous smears consisted of large clumps of cornified cells with scattered polymorphonuclear leukocytes. The smears that were recorded − + +, + + and + + −, were grouped because (a) only one smear was taken daily and a typical + + smear might have been missed, and (b) the exfoliated cells of the vaginal grafts were not expelled as readily as those from the intact vagina and relatively fewer + + smears were obtained although the histological condition of the epithelium indicated estrus.

OBSERVATIONS

Gross changes in the grafts

The grafts were usually not apparent through the skin until at least two weeks after they had been implanted. After one month they were usually about 5 mm. in diameter and after two months they were approximately 8–9 mm. in diameter. Not all of the grafts were measured during these stages. A few grafts, especially in mice of the A strain, tended to remain small in the cyclic animals until extrinsic estrogens were added. Six non-fistulated grafts in 3 hybrid hosts (A and C_{57} donors) were followed by daily measurements for 2 to 5 months beginning 51 days after the operation, at which time the grafts measured from 5×5 to 9.6×10 mm. No consistent differences existed between the grafts from either the A or C_{57} strain donors. Two months later the grafts measured from 1.2 to 1.5 cm. in diameter. Daily fluctuations in size were relatively small and were not associated with the estrous cycles as revealed by vaginal smears.

The grafts were distended with cornified cells in a viscous mucous-like fluid which could be readily removed by slight pressure when the grafts were first opened (Figs. 3, 4, 5). After the grafts were opened they became smaller and never regained their pre-operative size. The fistulas occasionally healed and were re-established by a second operation but the animals were usually discontinued. Careful daily probing of the fistula at the time smears were obtained usually maintained the fistula until it had healed to the skin. The injection of 16.6 μg. of estradiol benzoate facilitated the maintenance of fistula while the wound was healing.

A number of animals were used to develop methods and procedures prior to designing the experiment. The preliminary experience resulted in comparatively fewer losses of grafts due to accidents during the course of the

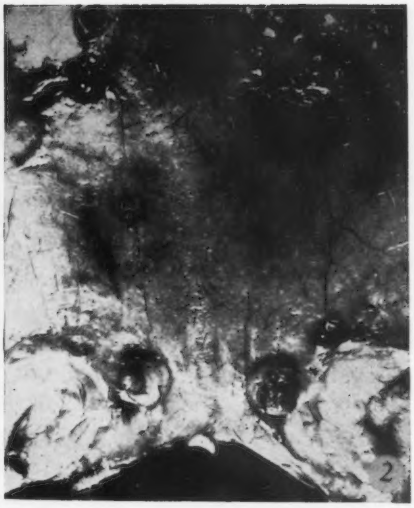

FIG. 1. Photograph of the shaved lower part of the body of a hybrid mouse (32, Table 9) bearing one transplanted vagina from each of the parental stocks. The grafts had been in place 379 days and had been examined daily for 208 days; 99 days prior to ovariectomy and 109 days during which estradiol benzoate was injected periodically. The mouse was killed 11 days after having been given a final injection of 16.6 μg. of estradiol benzoate.

FIG. 2. The appearance of the grafts shown above after they had been exposed upon removal of the skin. These grafts were loosely attached to the subjacent muscles. The mammary glands from the 4th and 5th inguinal pairs are located immediately lateral to the grafts.

FIGS. 3, 4, 5. Photomicrographs of the intact vagina and the transplanted C_{57} and A-strain vaginas, respectively, of the above mouse. All were lined by stratified epithelium. Leucocytes persisted in the lumen of the A-strain graft although they were not found in the area of epithelium shown.

experiment. Few grafts had fistulas that closed, that were too small to permit adequate entry for the determination of the nature of the exfoliated cells, or that prolapsed because of inadequate fixation after a large fistula had been prepared.

Strain differences in the response of the vagina

The strain differences in the responsiveness of the vaginal mucosa were determined by the evaluation of the responses to single subcutaneous doses of estrogen (Table 1). The range of strain differences noted earlier (4) was smaller in this experiment, probably because of the different criteria used to evaluate positive responses, but the order of responses were the same as noted earlier. The sensitivity of the vaginal mucosa of the C_{57} strain was

TABLE 1. SUMMARY OF THE RELATIVE RESPONSIVENESS OF THE INTACT VAGINAS OF OVARIECTOMIZED MICE OF THE A AND C_{57} STRAINS AND OF THEIR F_1 HYBRIDS (AC) WHEN GIVEN ESTRADIOL BENZOATE. (COMPILED FEOM TABLES 2–5)

μg. of estrogen	A*		C_{57}		AC	
	No. of tests	% positive	No. of tests	% positive	No. of tests	% positive
0.25	28	96	14	100	47	100
0.125	41	77	26	100	73	92
0.0625	32	56	37	95	88	75
0.0469	12	25	24	87	36	61
0.039	—	—	—	—	8	87
0.03125	—	—	—	—	4	50

* Includes all smears read as late proestrus (−++), estrus (+++), or early post estrus (+++−).

greater than that of the hybrids (AC). The A-strain mice were least responsive. Only 56% of 32 tests conducted in mice of the A strain given 0.0625 μg. of estradiol benzoate were positive whereas 95% of the 37 tests performed with C_{57} mice were positive (Table 1). The amount of estradiol benzoate required for a 50% response of the vaginas in C_{57} mice and hybrids was probably near 0.0312 μg. Seventy-five per cent of the hybrid mice responded to 0.0625 μg. The minimal doses required for the response of the intact vaginas were not determined because the amount of hormone was less than that required by the grafts. The relative responses of the grafts were the more important part of this investigation.

Comparison of the response of the intact and transplanted vaginas

The intact vaginal epithelium was always more responsive to the injected estrogen than was the vaginal mucosa of the transplants as determined by the smears of the exfoliated cells (Tables 2, 3), and as indicated by the sections of the vaginal mucosa studied at the termination of the experiment (Figs. 3, 4, 5). The transplanted vaginas required 2 or more times the amount of estrogen for responses comparable to those of the

TABLE 2. RESPONSIVENESS OF THE INTACT AND TRANSPLANTED VAGINAS FROM MICE OF THE A AND C_{57} STRAINS AND OF THE HYBRIDS (AC) TO INJECTED ESTRADIOL BENZOATE

Strain and No. of mice	μg. of estrogen	Intact vaginas		Transplanted vaginas			
				Right		Left	
		No. of tests/ No. ++*	% positive	No. of tests/ No. ++	% positive	No. of tests/ No. ++	% positive
A(7)	0.25	28/27	96	23/16	69	26/19	73
	0.125	41/32	78	30/12	40	38/14	37
	0.0625	32/18	56	20/5	25	27/10	36
C_{57}(7)	0.25	14/14	100	14/11	78	14/10	71
	0.125	26/26	100	26/19	73	24/12	50
	0.625	37/35	95	37/20	54	32/16	50
	0.0469	24/21	87	24/10	41	20/10	50
AC(11)	0.25	26/26	100	26/19	73	26/20	77
	0.125	28/25	89	28/20	71	28/14	50
	0.0625	38/22	84	36/15	42	38/20	53
	0.0469	19/11	58	19/7	37	19/11	58
	0.0389	5/4	80	5/1	20	5/0	0

* Includes vaginal smears recorded as −++, ++, ++−.

intact vaginas. The responses of the transplants from hosts of the same strain were similar or showed either one graft and then another with slightly greater responsiveness. The vaginal grafts in the mice of the C_{57} strain, which was most susceptible to estrogen, were relatively no more susceptible than those of the hybrid group. The vaginal grafts in mice of the A strain were relatively less responsive than the intact vaginas (50% level of response required more than 0.125 μg. of estradiol) whereas the intact vagina required about 0.0625 μg. of estradiol benzoate (Table 2).

The intact vaginal epithelium was also more responsive, on the average, to intrinsic hormones produced by the intact ovaries than was the epithelium of the vaginal transplants (Tables 4, 5). The transplanted vaginas

TABLE 3. SUMMARY OF THE RESPONSES OF THE INTACT VAGINAS OF TWELVE HYBRID MICE (AC) AND OF THE TRANSPLANTED VAGINAS OF THE PARENTAL STRAINS TO ESTRADIOL BENZOATE (12 MICE USED REPEATEDLY)

μg. of estrogen	Intact		Transplanted			
	AC		C_{57}		A	
	No. of tests/ No. ++*	% positive	No. of tests/ No. ++	% positive	No. of tests/ No. ++	% positive
0.5	4/4	100	4/3	75	4/3	75
0.25	21/21	100	21/19	90	21/16	76
0.125	45/43	92	45/37	82	45/26	58
0.0625	50/42	79	50/28	56	50/15	30
0.0469	17/11	65	17/6	35	17/2	12
0.0398	3/3	100	3/0	0	3/0	0
0.0312	4/2	50	4/0	0	4/0	0
Total	144/126		144/93		144/62	

* Includes vaginal smears recorded as −++, ++, ++−.

TABLE 4. RESPONSES OF THE INTACT VAGINAS OF 13 HYBRID MICE AND OF THE TRANSPLANTED VAGINAS OF MICE FROM THE PARENTAL STRAINS TO INTRINSIC HORMONES

No. of mouse	AC		C_{57}		A	
	No. of tests/ No. ++	Cornified cells days	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, day
22	17/17	46	17/16	28	17/7	8
23	16/16	38	16/6	10	16/15	18
24	17/17	49	17/14	32	17/9	16
28	5/5	20	5/4	8	5/3	6
29	16/16	72	16/13	49	16/14	51
30	15/15	63	15/15	48	15/15	46
31	7/7	27	7/0	—	7/0	—
32	7/7	28	7/7	15	7/4	7
40	21/21	52	21/9	19	21/18	25
41	21/20	41	21/17	34	21/15	22
44	16/16	35	16/12	17	16/7	9
46	19/18	44	19/13	22	19/15	26
49	17/17	41	17/10	15	17/15	26
Total 13	194/192	586	194/136	297	194/137	260
Average	14.8/14.7	45	14.8/10.6	22.8	14.8/10.6	20

showed extensive vaginal cornification (−++, ++, or ++− response) only about half as frequently as did the intact vaginas (Table 5). The number of days that the transplants revealed estrous smears averaged less than half that for the intact vaginas. Again the relative responses of the intact vaginas and of the autologous grafts (A into A, C_{57} into C_{57} and AC into AC) were strikingly similar to the responses of the same groups to extrinsic hormones (Table 5). The intact vaginas almost always showed more periods of vaginal cornification and a greater duration of vaginal cornification.

Strain differences in the response of the transplanted vaginas in hybrid hosts

The 144 tests done on the hybrid hosts (Table 3) indicated that the 50% level of response was probably slightly below 0.04 μg. of estradiol benzoate. The 50% level of response, as determined from 50 tests, was

TABLE 5. SUMMARY OF THE RESPONSES OF THE INTACT AND TRANSPLANTED VAGINAS TO INTRINSIC HORMONES OF CYCLIC MICE OF THE FOUR GROUPS. (FIGURES GIVEN AS AVERAGES OF THE GROUPS AND CAN ONLY BE COMPARED HORIZONTALLY)

Group		No. of mice	Intact		Transplanted			
Donor	Host		No. of tests/ No. ++*	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days
A	A	7	8.7/ 8.6	25.6	7.8/ 4.8	9.4	8.6/ 3.7	8.9
C_{57}	C_{57}	7	5/ 5	16.9	5/ 3	7	5.2/ 3.8	8
AC	AC	11	11.8/11.6	31.7	11.8/ 6.5	16.1	11.8/ 7.3	14.7
					C_{57}		A	
A+C_{57}	AC	13	14.8/14.7	45	14.8/10.6	22.8	14.8/10.6	20

* Includes smears recorded as −++, ++, ++−.

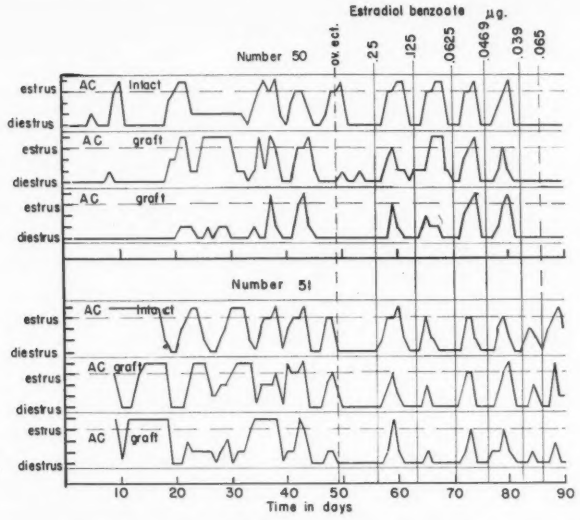

CHART 1. Diagram of responses of intact and transplanted vaginas of two mice (50 and 51 in Table 8) during estrous cycles and subsequent to ovariectomy and the injection of estradiol benzoate. The number of cycles of mouse 50 does not correspond to the number listed in Table 7 because some of the cycles occurring shortly after the transplants were fistulated were not included on the chart. Animal 50 shows unusual variability of response of the vaginal mucosa at the 3 sites. The charting is as follows: top line, early postestrus; second line, estrus; third line, late proestrus; fourth line, early proestrus or late postestrus and bottom line, diestrus.

slightly less than 0.0625 µg. for the transplanted C_{57} vaginas (95 tests were done at the critical area). The relative order of response of the transplanted A and C_{57} vaginas in the hybrid hosts was the same as for the intact vaginas (56% of the A mice responded to 0.0625 µg. in 32 tests whereas

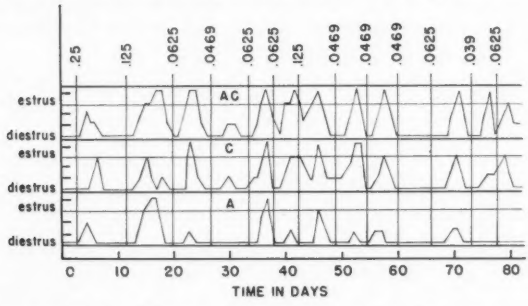

CHART 2. Diagram of the responses of the mucosa of the intact and of the transplanted vaginas of the parental strains to injected estradiol benzoate (one injection in 0.05 cc. sesame oil). The amounts of hormone injected are listed along the upper border. The vaginal smears were recorded as described in Chart 1, and represent mouse 44, Table 9.

TABLE 6. RESPONSIVENESS OF THE INTACT AND TRANSPLANTED VAGINAS OF SEVEN MICE OF THE A STRAIN TO INJECTED ESTRADIOL BENZOATE

No. of mouse	Intact		Right		Left	
	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days
1	16/14	32	16/4	7	16/7	8
5	17/12	33	—	—	17/8	13
6	27/19	44	27/12	31	27/15	28
8	17/12	34	17/8	15	27/3	4
11	14/9	20	4/1	1	14/8	13
17	10/5	13	3/2	3	10/4	10
85	12/7	22	12/6	12	—	—
Total	113/78	198	79/33	69	111/45	76
Average	16.1/11.1	28.3	13.1/5.5	11.5	18.5/7.5	12.7

95% of the C_{57} responded to the same dose). The differences between the transplanted A strain and C_{57} strain vaginas were evident, however, at all dose levels except the highest and the subthreshold doses (Table 3). This adds further evidence of the differences in the intrinsic capacity of the vaginal epithelium of the mice of the two strains in the same environment.

In only one hybrid host did a transplanted A-strain vagina respond more frequently than did the C_{57} strain vagina (23, Table 9). This mouse was followed for only 7 tests, two of which were at the highest levels (0.5 μg. estradiol benzoate) and two at the lowest levels (0.0312 μg.). Also, the number of days of vaginal cornification of the A-strain grafts exceeded that of the contralateral C_{57} strain grafts only in this one instance. The average number of days per cycle was about the same for both the A and C_{57} strain transplants with a tendency for the C_{57} to be slightly longer. The average period of cornification of the grafts was always less than for the intact vaginas (Tables 6–9).

The mice of the A strain were the most difficult to work with and a number of the grafts deteriorated during the course of the experiment. The number of tests conducted, using the grafts on the right and left

TABLE 7. RESPONSIVENESS OF THE INTACT AND TRANSPLANTED VAGINAS OF 7 MICE OF THE C_{57} STRAIN TO INJECTED ESTRADIOL BENZOATE. THE DOSES OF ESTROGEN ARE LISTED IN TABLE 2

No. of mouse	Intact		Right		Left	
	No. of tests/ No. ++	Days	No. of tests/ No. ++	Days	No. of tests/ No. ++	Days
4	15/15	83	15/12	36	15/9	36
8	13/12	39	13/11	39	—	—
9	17/17	48	17/10	22	17/8	12
10	14/14	38	14/7	19	14/6	16
11	14/11	29	14/4	11	—	—
12	14/14	36	14/12	31	14/12	31
15	14/13	45	14/5	16	14/11	42
Total	101/96	318	101/51	174	74/46	137
Average	14.4/13.7	45.4	14.4/7.3	24.9	14.8/9.2	27.5

TABLE 8. RESPONSIVENESS OF THE INTACT AND TRANSPLANTED VAGINAS OF ELEVEN HYBRID MICE (AC) TO INJECTED ESTRADIOL BENZOATE. (SEE TABLE 2 FOR AMOUNTS OF ESTROGEN)

No. of mouse	Intact		Right		Left	
	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days	No. of tests/ No. ++	Cornified cells, days
43	4/4	10	—	—	4/0	0
58	7/7	21	7/2	3	7/5	11
59	7/6	14	7/3	5	7/2	2
60	7/7	30	7/4	16	7/4	9
50	6/5	17	6/5	12	6/3	6
51	7/7	17	7/7	13	7/4	4
55	17/14	33	17/12	37	17/12	26
95	17/15	27	17/7	9	17/6	6
97	14/11	25	14/7	13	14/10	22
98	15/11	24	15/5	7	15/11	30
100	15/11	28	15/9	18	15/8	14
Total	116/98	246	112/61	133	116/65	130
Average	10.5/8.9	22.4	11.2/6.1	13.3	10.5/5.9	11.8

sides, therefore differ (Table 6). Infection and abscess formation were the causes of deterioration. The inbred mice of this strain are not particularly hardy and comparatively few of them were continued for a total of over 25 tests (Tables 5 and 6). The grafts that responded most consistently in the intact mice usually responded most consistently in the ovariectomized mice given extrinsic hormones.

The grafts in C_{57}-strain mice also responded similarly to intrinsic and injected hormones (Tables 5 and 7). Comparable responses were also observed for the hybrid mice (Tables 5 and 8).

The relative responsiveness of the homologous grafts in the hybrid mice to intrinsic hormones (Table 4) indicated very little difference in the responsiveness of the tissues derived from the A or C_{57} donors. The number

TABLE 9. RESPONSIVENESS OF THE INTACT VAGINAL MUCOSA OF 12 HYBRID MICE AND THE TRANSPLANTED VAGINAS FROM THE PARENTAL STRAINS TO INJECTED ESTRADIOL BENZOATE

No. of mouse	Intact		Right (C_{57})		Left (A)	
	No. of tests/ No. ++	Days	No. of tests/ No. ++	Days	No. of tests/ No. ++	Days
22	7/6	22	7/4	14	7/2	3
23	7/6	26	7/2	7	7/5	17
28	13/12	34	13/10	27	13/10	22
29	12/11	31	12/9	23	12/6	16
30	13/11	34	13/12	34	13/6	17
31	13/13	35	13/5	4	13/2	2
32	12/12	49	12/9	16	12/5	6
40	14/13	36	14/9	26	14/8	14
41	14/10	31	14/7	22	14/4	9
44	14/12	32	14/11	20	14/4	7
46	13/10	21	13/7	11	13/6	9
49	12/10	36	12/8	20	12/3	9
Total	144/126	387	144/93	224	144/62	131
Average	12/10.5	32.3	12/7.7	18.7	12/5.2	10.9

of estrous responses for the grafts from the A and C_{57} strain averaged 10.6 for both and the number of days that cornified cells were present were almost the same (20 and 22.8 days). In several instances the grafts from the A-strain donors were more responsive than those from C_{57} donors (Numbers 23, 29, 40, 46, 49—Table 4). The hormones produced by the hybrid host induced comparable responses in the grafts from both donors.

In contrast the response of the A-strain grafts in the same hybrid host, after castration and when given extrinsic hormones, was lower with one exception (Number 23) than that of the C_{57}-strain grafts (Table 9). The number of estrous tests averaged 5.2 for the A-strain grafts and 7.1 for the C_{57}-strain grafts. The corresponding days of vaginal cornification average 10.9 and 18.7 respectively.

The relatively greater responsiveness of the parental strain grafts in cyclic hybrid mice (Table 4) than of the hybrid grafts in the hybrid hosts (Table 5) is striking; during only 71 and 80 of the 130 cycles did the grafts from hybrid donors show positive smears, whereas 136 and 137 of the C_{57}- and A-strain grafts gave positive responses in the 194 cycles studied.

The average responsiveness to injected estrogen of the transplanted vaginas of parental strains and of the hybrids in the hybrid hosts (Tables 8 and 9) indicate that the hybrid tissues are slightly more responsive. The data presented in this way, however, are not suitable for comparison because the differences in the amounts of hormone injected into the mice of of the two different groups. About 0.0625 μg. of estradiol benzoate were required to induce a 50% response in the transplanted hybrid and C_{57} vaginas and about 0.125 μg. for the A-strain tissues (compare Tables 2 and 3).

DISCUSSION

The differences in the responsiveness of the transplanted and host vaginal tissue, even when the transplants were isologous, cannot be explained adequately. The transplants may not have been as well vascularized as the intact tissues although they were placed just medial to the fourth or anterior inguinal pair of mammary glands. Usually the blood supply was provided by contact with the dermis. Some grafts were attached to muscles or muscular fascia as well as the loose mammary fascia. Transplanted vaginas were well vascularized when they were examined at the time the fistulas were established and at necropsy. Nevertheless the grafts seemed less vascular than the intact vaginas and seemed to show less hyperaemia after estrogen treatment. The impressions were subjective, however. The grafts were examined only through the skin during the first one or two months.

Several grafts were measured daily for periods of 2 to 5 months. They revealed no periodic changes in size that could be correlated with the cyclic exfoliated epithelial cellular changes of the intact vaginas. During

this time the grafts increased in size from an average of approximately 8×8 mm., as measured through the skin, to about 14×15 mm. The grafts of the A and C_{57} donors were not significantly different in the total sizes attained.

Estrogen was applied directly to the lumen of the transplants in several instances and to the intact vagina. This sensitive test for estrogen (12) that was used to differentiate direct and indirect estrogens (13) was not practical in this instance because of the failure of the transplant to discharge the exfoliated cells as rapidly as the intact vagina. Exfoliated cells could not be used as a criterion for response to determine whether the grafts responded less readily because (a) less circulating hormone reached the responsive cells, or (b) whether transplantation of the tissue irreversibly reduced the subsequent responsiveness of the tissue for other and unknown reasons. The consistency of the results, however, in all groups showed that the transplants were not as responsive, whatever the cause, as were the intact tissues.

The intact vaginas of the mice of all groups consistently responded more frequently to intrinsic hormones than did the transplanted vaginas (Table 5). The number of days during which positive smears (late proestrus, estrus and early postestrus) were obtained also was greater for the intact vaginas by a ratio of about 3 to 2. Within the limits of the doses of estradiol benzoate used in these studies, and presumably the levels of hormone produced by the gonads, the time of onset of positive smears or their duration was not effected by the quantity of hormone. The responses in general tended to be all or none with the transplants responding at different thresholds. Similar observations have been made in mice given estrone at doses ranging from 0.5 to 2.5 μg. (14).

The hybrid mice are larger than mice of the parental strains. Although the animals used in this study were not weighed the hybrids from this cross weighed about 5 grams more than the parents. Differences in size, however, have not modified the vaginal response to estrone (15) and could not account for the differences in the vaginal response observed during this investigation.

The level of thyroid function has modified the response of the vagina to injected estrogens (16) and might account for strain differences in responses. The amounts of thyroxine produced by mice of different strains ranges appreciably (17). Mice of the A strain required less thyroxine to prevent thyroid hypertrophy during thiouracil treatment than did mice of the C_{57} strain (17). Strain differences in the hepatic storage of vitamin A (18) may be correlated with the sensitivity of the vaginal mucosa. The enthusiasm with which such variables or possible variables can be used to explain strain differences in responsiveness of the vaginal mucosa wanes when the most responsive and least responsive tissues retain their characteristic sensitivities in the same environment.

The experiments reported here were undertaken with the application of different amounts of estradiol benzoate in a single injection dissolved in 0.5 cc. of sesame oil. The length of time during which the threshold levels were maintained within the body under such a schedule of injections is unknown. A number of studies have been done on the effect of repeated injections on the response to estrogen (19). The strain differences in responsiveness may be related more closely to the duration of the maintenance of a threshold dose than to the concentration of hormone. The rate of response might be confused in an interpretation of threshold of response. For example, one animal might require a level of hormone x during a time y to produce a given response, whereas a second animal might require the same level of hormone for the interval $2y$ for a similar response. If such is true the differences in the responsiveness between mice given the more rapidly destroyed non-esterified estrogens might be greater than among those mice given the esters. Mühlbock (5) reported slightly greater strain differences in vaginal responsiveness among mice given estrone than were observed among mice given estradiol benzoate (4). The strain differences were less striking in the present study, probably because of differences in the criteria of evaluation of the smears. A completely cornified smear was not required because of the difficulty of getting such vaginal exfoliates from grafts in which drainage and slough were not as rapid as in the intact vagina. The strain differences in response were similar except in magnitude, however. The intrinsic strain differences in the vaginal responsiveness may be due not to differences in threshold but to differences in rate of responsiveness or a maintenance of threshold levels of estrogen for an interval long enough to permit a complete response.

The average duration of the positive smears of the intact vagina was about 3 days for all groups (Table 5). The isografts averaged only about 2 days or less of vaginal cornification per cycle (Table 5). Although the grafted vaginal tissues of the parental strains in the hybrid hosts responded two-thirds as frequently as did the intact vaginas the number of days of vaginal cornification of the transplant was only about one half as long (Table 4). The response of the A- and C_{57}-strain transplants to the intrinsic hormones of the hybrid hosts were similar. The same hosts, when ovariectomized and given extrinsic hormones showed differential responses between the A and C_{57} strain consistent with the differences in the response of the intact vaginas. It is therefore possible that strain differences in response are differences only to extrinsic hormones and not to the quality or balance of hormone existing in the body.

The potentiality for neoplasia in testis, lungs and the adrenal cortex is strain-limited and is present in the responding tissues rather than in the host's environment (7–10). The specific end organ, at least when transplanted at those ontogenic stages that have been studied, must be capable

of responding in a manner consistent with its genotype in an environment that is foreign to it. The capacity of certain genotypes to permit neoplastic change might well be looked upon as a specific quality. Apparently quantitative differences in response are also genetically determined and are manifest phenotypically at the level of an individual organ or tissue since the responses of the epithelium alone were measured in this study. The potentialities of the tissue, qualitatively as well as quantitatively, are resident in the tissue itself rather than in the environment.

The above observations, if extrapolated generally, would emphasize the multiple individualities of organisms. Some of these individualities may be direct phenotypic manifestations but some may be indirect, or due to ontogenic experiences that result in irreversible changes. Pfeiffer (20), for example, demonstrated that the testis transplanted into a newborn sib irreversibly modified the host's production of gonadotropin. It is not improbable that ontogenic experiences may be modifying some of the quantitative or qualitative responses of tissues to specific stimuli.

SUMMARY AND CONCLUSIONS

Strain differences in the responsiveness of the vaginal mucosa of mice from the A and C_{57} strains exist; mice of the A strain require larger amounts of estradiol benzoate.

Vaginas from young hosts of the A and C_{57} strain grow when transplanted subcutaneously in hybrid ($A \times C_{57}$) hosts. When subsequently opened and sutured to the skin to establish fistulas they yield exfoliated epithelial cells that indicate responsiveness to estrogens. Vaginal tissues of the parental strains and hybrid groups also grew when transplanted.

Subcutaneous grafts of vaginal tissue required about twice as much estradiol benzoate as did the intact vaginas for comparable responses.

Transplanted vaginal tissues of the A-donors required more estrogen than did those from the C_{57} donors when both were in the same host.

Transplanted vaginal tissues also responded to intrinsic hormones produced by the host's ovaries but less consistently than did the intact vaginas.

Strain differences in the responsiveness of the transplanted vaginal tissues of the parental strains (A and C_{57}) were not revealed in hybrid hosts with normal ovarian function. The intact ovaries induced comparable responses in the transplants from both strains.

The strain differences in the responsiveness of the vaginal mucosa are quantitative and presumably resident in the vaginal tissues.

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BLOOD PROTEIN-BOUND IODINE IN THE FOWL[1,2]

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EARLY studies of serum or plasma protein-bound iodine (PBI) showed it to be a reliable and valuable criterion of thyroid state in humans (1, 2). Subsequent studies have largely confirmed this observation, and, in the opinion of some clinicians, PBI "is probably the single most reliable test of thyroid function" (3).

In recent years, workers interested in the physiology of domestic mammals have studied blood PBI in relation to other characteristics of physiological and economic importance. Long *et al.* (4) found significant breed differences in PBI of dairy cattle, and also noted that calves had higher PBI levels than cows. Burns *et al.* (5) reported a highly significant correlation between PBI and metabolic rate in bulls. In beef calves, there is apparently an optimum PBI level associated with the best rates of gain (6), while in Hampshire swine, a significant negative correlation between PBI level and average daily gain has been reported (7). In a study of adrenal cortex-thyroid relationships in ketotic cows, Robertson *et al.* (8) found that high 17-hydroxycorticosteroid levels in plasma were associated with low plasma PBI values.

There are practically no data on avian PBI values in the literature. We have found only two reports on chickens, each based on only three birds. In one of these papers (9), a PBI range of 3.3 to 3.8 micrograms per 100 ml. of serum was reported; in the other (10), the mean value was 2.6 μg.%. Our research on blood PBI in the domestic fowl was initiated so that more extensive data on birds could be obtained, and with the expectation that PBI level would serve as a reliable index of thyroid activity in a variety of physiological and genetic studies with chickens.

MATERIALS AND METHODS

The method used for all PBI determinations was essentially that of Brown *et al.* (11). This method involves alkaline ashing of washed serum or plasma proteins, acid leaching of iodide from the ash, and colorimetric determination of the iodide by its catalytic effect

Received October 31, 1956.

[1] Portions of this study were conducted as part of a Northeast Regional Project (NE-6); a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds of the United States Department of Agriculture.

[2] Published as Miscellaneous Paper No. 256 with the approval of the Associate Director, Delaware Agricultural Experiment Station.

on the reduction of ceric ion by arsenious acid. Some modifications were introduced to allow for the use of 2-ml. portions of serum or plasma and to adapt the technique for use with the Evelyn colorimeter. Valuable suggestions of Mixner and Lennon (12) were followed with respect to treatment of reagent blanks and standard iodine tubes and in calculating PBI values from colorimetric readings (percent transmittance).

Chicken blood and duck blood were obtained by heart puncture; cattle blood was drawn from the jugular vein. In the early work, the serum was used; but after several comparisons of serum, oxalated plasma, and heparinized plasma from the same animal showed no differences in PBI, plasma was used exclusively. In most of the work, the serum or plasma was quick-frozen and stored at −10° F. This allowed bleeding of all experimental groups at approximately the same time and subsequent equal representation of all experimental lots in each PBI "run." Although no specific experiments were designed to test possible effects of frozen storage on PBI level, extensive observations indicated no effect.

The chickens in experiment 2 were hatched from our experiment station flocks and reared in electrically-heated battery cages. The birds were transferred at 4 weeks of age to larger unheated cages in the same room. Room temperature was maintained at 65°–70° F at all times, and the room was continuously illuminated by means of fluorescent lights.

Chickens and ducks received standard all-mash rations and water *ad libitum*. For the lot fed thiouracil in experiment 2, this drug was uniformly mixed in the mash, 0.1% by weight. Several investigators have shown that this level of thiouracil is non-toxic and has a maximum anti-thyroid effect when fed to chickens.

RESULTS AND DISCUSSION

Preliminary studies. PBI analyses for pullets and hens (Table 1) resulted in rather good agreement between duplicate plasma samples; however, the PBI values were much lower than those reported for normal mammals, with the exception of the opossum (10). At first we suspected some consistent error in our method. Subsequent PBI analyses for cattle,[3] rats, rabbits, and ducks (all performed in our laboratory by the same method) dispelled this suspicion. These data (Table 2), together with various mammalian data in the literature, show that in at least two avian species blood PBI level is characteristically and consistently much lower than in most mammals. It should be noted that the avian data were obtained during the winter months, when thyroid activity is relatively great.

We were concerned over the discrepancy between our avian PBI values and the higher ones previously reported (9, 10), but this concern was lessened by unpublished data kindly furnished from two other laboratories. Bumgardner and Shaffner (13), using the method of Brown *et al.* (11), found a mean PBI level of 1.09 μg.% in 50 New Hampshire chickens. Stutts and Kunkel (14), using the acid digestion-distillation method of Moran (15), found mean values of less than 1 μg.% in each of three different strains of chickens.

In view of the fact that growing Pekin ducks have a higher thyroid se-

[3] The cattle PBI data were obtained from experiments conducted as part of a Northeast Regional Project (NE-30) on dairy cattle physiology.

TABLE 1. PBI IN DUPLICATE PLASMA ALIQUOTS FROM FEMALE CHICKENS OF THREE BREEDS

Breed	Approx. age (months)	PBI (μg. %)
New Hampshire #1	8	1.48 1.43
#2	8	1.08 0.98
#3	8	1.13 1.23
White Plymouth Rock #1	8	1.17 1.07
#2	8	1.12 1.42
#3	8	1.27 1.17
#4	8	1.12 1.12
#5	8	1.22 1.46
#6	8	0.88 0.98
#7	8	1.12 1.02
White Leghorn #1	20	1.22 1.07
#2	20	1.03 1.03
#3	20	1.22 1.22

Mean of all determinations $\pm$SE = 1.16 $\pm$ 0.03.

TABLE 2. REPRESENTATIVE PLASMA PBI VALUES FOR DIFFERENT SPECIES

	No.	Age	Month	PBI, μg. % (mean $\pm$ S.E.)
N.H. pullets	3	8 mo.	Nov.	1.22 $\pm$ 0.07
W.P.R. Pullets	7	8 mo.	Nov.	1.15 $\pm$ 0.04
W.L. hens	3	20 mo.	Nov.	1.13 $\pm$ 0.04
Pekin ducks	6	6 wk.	Dec.	1.17 $\pm$ 0.08
	6	10 wk.	Jan.	1.14 $\pm$ 0.06
	4	14 wk.	Feb.	1.49 $\pm$ 0.10
Holstein cows	8	2–8 yr.	Jan.–June	4.46 $\pm$ 0.33
Holstein heifers	8	3–6 mo.	Jan.–June	5.06 $\pm$ 0.22
Guernsey cows	6	3–7 yr.	Jan.–Apr.	3.91 $\pm$ 0.22
Guernsey calves	6	3–6 mo.	Jan.–Apr.	6.67 $\pm$ 0.26
Albino rats	3	mature	Apr.	3.50 $\pm$ 0.12
Rabbits	2	young	Mar.	2.16 $\pm$ 0.34

TABLE 3. EFFECT OF AMBIENT TEMPERATURE ON PLASMA PBI OF WHITE PLYMOUTH ROCK COCKERELS*

Weeks	Heated room		Unheated pen	
	Mean temp. (deg. F.)	PBI (μg. %)	Mean temp. (deg. F.)	PBI (μg. %)
0			34	1.23
1	72	1.71	41	1.66
2	72	1.73	40	1.66
3	75	1.51	35	1.11
4	76	1.17	42	1.09
Means (1–4 wk.)	74	1.53 ±0.14†	40	1.38 ±0.14†

* 5 birds on each treatment.
† Standard error.

cretion rate than chickens (16, 17), it is particularly interesting to note that PBI levels in the ducks used in these studies were no higher than those in chickens. This observation indicated that PBI level in birds might not reflect relative thyroid activity.

Experiment 1. This experiment was designed to test the effect of ambient temperature on PBI level in the fowl. Ten cockerels housed in an unheated pen were bled, then 5 randomly-selected birds were moved to cages in a heated room; the other 5 remained in the colder environment. All birds were bled at weekly intervals for 4 weeks. The pertinent data are shown in Table 3. It is evident that the difference in ambient temperature did not cause a difference in PBI level; the over-all mean values were not significantly different by "t" test. Hoffmann and Shaffner (18) demonstrated that thyroid secretion rate, as determined by the goiter-prevention method, was distinctly higher in chickens kept in a cool environment than in those maintained in a warm room. Thus the data of our experiment suggest that PBI in the fowl does not fluctuate with changes in thyroid activity.

Experiment 2. The effect of thiouracil on plasma PBI was tested in the manner indicated in Table 4. Eight males and 8 females were bled, then 4 birds of each sex were placed on the thiouracil treatment. All birds were

TABLE 4. EFFECT OF THIOURACIL (0.1% IN FEED) ON PLASMA PBI OF NEW HAMPSHIRE CHICKENS*

Weeks	Age (wk.)	PBI (μg. %)	
		Control	Thiouracil
0	9	1.11	
1	10	1.28	1.33
2	11	1.32	1.55
3	12	1.39	1.27
	Means (1–3 wk.)	1.33 ±0.07†	1.38 ±0.07†

* 4 males and 4 females on each treatment.
† Standard error.

then bled at weekly intervals for 3 weeks. Analysis of the over-all mean PBI values by "t" test showed no significant difference between treatments. As a check on the antithyroid potency of the thiouracil, the thyroid glands of all birds were carefully removed and weighed at the end of the experiment. The mean thyroid weight in the treated group was 26.2 mg./100 gm. body weight as compared to 10.2 mg./100 gm. in the controls.

SUMMARY

Blood protein-bound iodine (PBI) was determined in several species by a modification of the method of Brown, Reingold and Samson. It was found that PBI level in chickens and ducks was consistently and characteristically much lower than in mammals.

Experimental alteration of thyroid activity did not produce changes in blood PBI level in chickens. PBI is not a good criterion of thyroid activity in the fowl.

Acknowledgments

We are grateful to Miss Joanne Barczewski and Mr. Carroll Mumford for technical assistance; and to Drs. C. R. Richards, O. J. Abbott, J. H. McClendon, and G. F. Somers for valuable suggestions and critical appraisal of the manuscript.

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COMPOUNDS WITH ANTIESTROGENIC ACTIVITY *IN VITRO*[1]

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THE human placenta contains a DPN-linked isocitric dehydrogenase which is stimulated by the addition of estradiol *in vitro* (1–4). The enzyme was localized in the non-particulate fraction of homogenized placenta by differential ultracentrifugation in 0.25 *M* sucrose (2). Evidence that the estrogen-sensitive enzyme is isocitric dehydrogenase came from experiments with each of the tricarboxylic acids as substrates and with *trans*-aconitate and fluorocitrate as specific enzyme inhibitors (3). Further experiments showed that estrone, equilin and equilenin have the same enzyme-stimulating property possessed by estradiol-17β. However, a great many other compounds tested, including estradiol-17α, estriol, progesterone, 17α-ethinylestradiol, testosterone, 19-nortestosterone, stilbestrol, cortisone, cortisol, and cholesterol, had little or no effect, no more than one one-thousandth as much as estradiol-17β (5).

The results of kinetic experiments with this system are consistent with the hypothesis that estradiol unites with the protein to convert an enzymatically inactive form to an active form (6, 7). The apparent equilibrium constant for the dissociation reaction, calculated from two independent experiments, one measuring DPN reduction (6) and the other measuring α-ketoglutarate production (7) were in close agreement, 2.8×10^{-7} and 5×10^{-7} moles per liter, respectively.

Professor Ernst Bárány of the University of Uppsala, Sweden, kindly provided us with a number of stilbestrol homologues and related compounds which he had synthesized and tested for antiestrogenic activity in the rat *in vivo* (8). The present paper reports experiments in which these compounds were tested for estrogenic activity *in vitro*, using the placental isocitric dehydrogenase system.

MATERIALS AND METHODS

Term placentas were obtained immediately after delivery and were used within five minutes. The methods of homogenizing the placenta and of preparing a particle-free fraction by ultracentrifugation have been described previously (2). The incubations of

Received October 31, 1956.

[1] This investigation was supported by grants from the Charles A. King and Marjorie Kind Fund and by research grant C2400 from the National Institutes of Health, United States Public Health Service.

the particle free supernatant ($S_{57,000}$) were carried out at 37° C in 30 ml. beakers shaken at 45 cycles per minute in a Dubnoff incubator. Each beaker contained 1.0 ml. of $S_{57,000}$, 1.0 ml. of a buffer containing 30 micromoles K^+, 10 micromoles Mg^{++}, 20 micromoles Cl^- and 20 micromoles of phosphate buffered at pH 7.4, 3.0 micromoles of citrate, *cis*-aconitate or d-isocitrate, or 6.0 micromoles of dl-isocitrate, 0.75 micromoles of DPN, estrogens and antiestrogens added as aqueous suspensions prepared by homogenization, and water to a total of 3.0 ml. Incubations were continued for one hour, then aliquots were analyzed for citric and α-keto acids and for total nitrogen. Citric acid was estimated by the Elliott modification of the method of Natelsbn, Pincus and Lugovoy (9) and α-keto acids by our own modification of the method of Friedemann and Haugen (10). Nitrogen analyses were made by digestion and nesslerization and all data are expressed in terms of the mg. nitrogen of the non-particulate fraction used.

RESULTS

The results of experiments with stilbestrol and with 1, 3 di-*p*-hydroxyphenyl propane, Bárány's compound C-3, are presented in Table 1. The figures given in columns 1, 3 and 4 in this and in Table 2 were obtained by chemical analysis and represent micromoles of α-ketoglutarate produced

TABLE 1. EFFECTS OF STILBESTROL AND 1, 3 DI-*p*-HYDROXYPHENYL PROPANE (C-3) ON THE PLACENTAL ISOCITRIC DEHYDROGENASE SYSTEM

Substance added	Final conc. in incubation medium, μg./ml.	Estradiol-17β added, μg./ml.			
		0	0.01	0.1	1.0
Stilbestrol	0	0.23	0.34*	0.45	0.54
	0.33	0.19	—	0.41	0.55
	3.3	0.19	0.23*	0.32	0.48
	33.0	0.09	0.02*	0.15	0.26
Compound C-3	0	0.19	0.34*	0.49	0.58
	0.33	0.18	—	0.47	0.60
	3.3	0.17	0.27*	0.44	0.56
	33.0	0.07	0.12*	0.28	0.45

* Micromoles DPN reduced/mg. N/15 min. measured spectrophotometrically at 340 mμ; all other figures are micromoles α-ketoglutaric acid produced/mg. N/hr. Each number is the mean of 12 determinations.

TABLE 2. EFFECTS OF 1, 6 DI-*p*-HYDROXYPHENYL HEXANE (C-6) AND 1, 10 DI-*p*-HYDROXYPHENYL DECANE (C-10) ON THE PLACENTAL ISOCITRIC DEHYDROGENASE SYSTEM

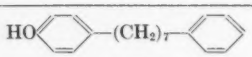
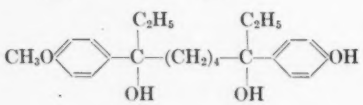
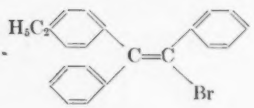
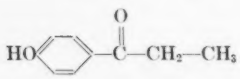
Substance added	Final conc. in incubation medium, μg./ml.	Estradiol-17β added, μg./ml.			
		0	0.01	0.1	1.0
Compound C-6	0	0.31	0.34*	0.55	0.77
	0.33	0.31	—	0.68	0.81
	3.3	0.30	0.31*	0.61	0.92
	33.0	0.20	0.28*	0.43	0.68
Compound C-10	0	0.26	0.34*	0.67	0.69
	0.33	0.23	—	0.60	0.76
	3.3	0.22	0.32*	0.58	0.75
	33.0	0.22	0.41*	0.69	0.80

* Micromoles DPN reduced/mg. N/15 min., measured spectrophotometrically at 340 mμ; all other figures are micromoles α-ketoglutaric acid produced/mg. N/hr. Each number is the mean of 12 determinations.

per hour per mg. N in the incubation medium. The results in the second column were obtained by spectrophotometric analysis and represent micromoles of DPN reduced per 15 minutes per mg. N. The data in this and in the subsequent tables were examined statistically by an analysis of variance.

The addition of estradiol-17β alone (first row) significantly increases the rate of the isocitric dehydrogenase reaction. Stilbestrol alone (column 1), or in combination with estradiol (columns 2, 3, and 4) significantly de-

TABLE 3. EFFECTS OF OTHER COMPOUNDS TESTED FOR ESTROGENIC AND ANTI-ESTROGENIC ACTIVITY IN THE PLACENTAL ISOCITRIC DEHYDROGENASE SYSTEM

Compound added		Final conc. in incubation medium, μg./ml.	Estradiol-17β added, μg./ml.	
			0	0.1
C-7		0	.29	.51
		33	.24	.45
5636		0	.31	.53
		33	.33	.48
LN-107		0	.39	.66
		33	.38	.64
5647		0	.17	.40
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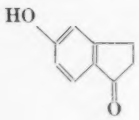
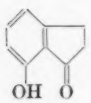
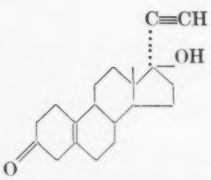
5643		0	.26	.48
		33	.34	.49
5642		0	.21	.52
		33	.30	.63
SC4642		0	.16	.47
		1	.16	.49
		10	.18	.50

All figures represent micromoles α-ketoglutaric and produced/mg. N/hr; each is the mean of six or more determinations.

creases the rate of the reaction. Higher concentrations of stilbestrol inhibit the reaction more effectively than lower concentrations and stilbestrol inhibition is more effective at lower concentrations of estradiol. The inhibition of estradiol stimulation by stilbestrol is therefore a competitive one.

Compound C-3 is an analogue of stilbestrol containing three instead of two carbon atoms between the phenolic rings and lacking both the double bond and the two ethyl groups of stilbestrol. It is similar to benzestrol, which is almost as effective an estrogen *in vivo* as stilbestrol. Compound C-3 significantly decreases the activity of the isocitric dehydrogenase system in the absence of added estradiol and at high concentrations competitively inhibits the stimulation of the enzyme by estradiol. The inhibition by C-3 is somewhat less marked than that produced by stilbestrol.

Compounds C-6 and C-10 (Table 2) are stilbestrol analogues comparable to C-3 but with six and ten carbon atoms, respectively, in the chain between the two phenolic rings. The distance between the hydroxyl groups in the extended forms of these molecules is approximately 40% greater in C-6 and 80% greater in C-10 than in stilbestrol. Compound C-6 has some inhibitory effect on the isocitric dehydrogenase system, but the inhibition is much less than that produced by stilbestrol. The statistical analysis shows that this inhibition does not quite reach the 5% level of confidence. Compound C-10 has neither an inhibitory nor a stimulatory effect on the enzyme system at the concentrations studied.

Seven other compounds, each of which has one or more chemical features in common with stilbestrol or estradiol, were tested for estrogenic and antiestrogenic activity using the isocitric dehydrogenase system (Table 3). None of these had a marked stimulatory or inhibitory effect on the enzyme. Compounds 5642 and 5643 had slight estrogenic effects and compound C-7 had a very slight inhibitory effect, but these were not statistically significant. Compound LN-107, similar to the unsubstituted triphenyl chloroethylene reported to be slightly estrogenic, had neither estrogenic nor antiestrogenic activity in this system.

TABLE 4. EFFECTS OF ESTRIOL AND ESTRADIOL-17α ON THE PLACENTAL ISOCITRIC DEHYDROGENASE SYSTEM

Substance added	Final conc. in incubation medium, μg./ml.	Estradiol-17β added, μg./ml.		
		0	0.1	1.0
Estriol	0	.22	.42	.47
	0.33	.29	.41	.47
	3.3	.34	.38	.44
	33	.38	.35	.40
Estradiol-17α	0	.22	.40	.41
	0.33	.16	.30	.36
	3.3	.13	.24	.30
	33	.12	.17	.25

All figures represent micromoles α-ketoglutaric acid produced/mg. N/hr; each is the mean of eight determinations.

Similar experiments were performed using estradiol-17α, estriol, progesterone and cortisone, alone and in combination with estradiol-17β. Estradiol-17α (Table 4) is a competitive inhibitor of estradiol-17β in this system and is about as effective in this respect as is stilbestrol. Estradiol-17α added alone (column 1) inhibits isocitric dehydrogenase and, when added with estradiol-17β, decreases the stimulatory effect of the latter (columns 2 and 3).

Estriol, in contrast to the preceding compounds, is slightly estrogenic when added alone (column 1, Table 4). This confirms our previous finding (5) in which only one level of estriol, 10 μg. per ml., was used. Thirty-three micrograms of estriol are less effective in stimulating the isocitric dehydrogenase system than 0.1 μg. of estradiol-17β. However, when estriol is added together with estradiol-17β, it decreases the stimulatory effect of that compound (columns 2 and 3, Table 4).

Progesterone and cortisone (Table 5) provide examples of steroids which have no apparent effect, either stimulatory or inhibitory, on the placental isocitric dehydrogenase.

TABLE 5. EFFECTS OF PROGESTERONE AND CORTISONE ON THE PLACENTAL ISOCITRIC DEHYDROGENASE SYSTEM

Substance added	Final conc. in incubation medium, μg./ml.	Estradiol-17β added, μg./ml.		
		0	0.1	1.0
Progesterone	0	.20	.41	.54
	0.33	.17	.41	.52
	3.3	.22	.46	.50
	33.0	.18	.43	.52
Cortisone	0	.28	.49	.56
	0.33	.25	.49	.53
	3.3	.29	.49	.57
	33.0	.30	.49	.56

All figures represent micromoles α-ketoglutaric acid produced/mg. N/hr; each is the mean of six determinations.

DISCUSSION

The results of these experiments suggest that estrogens and related compounds have two distinct properties with respect to the isocitric dehydrogenase: an ability to combine with the enzyme, and an ability to activate the enzyme after combination has occurred. The marked effect of estradiol-17α, stilbestrol and C-3 as competitive inhibitors of the estradiol-17β activation suggests that they have a strong ability to combine with the enzyme but little or no ability to activate it. Estriol has a greater enzyme-activating effect than any of these three. Bárány had found (8) that C-6, but not C-3, when applied topically prevents the estrogen-induced proliferation and cornification of the vaginal epithelium of rats.

The fact that stilbestrol, C-3, C-6, C-7 and C-10 can be arranged in a series of decreasing inhibitory effect which parallels the chain length, i.e.,

the distance between the terminal OH groups on the phenolic rings, suggests that this length is of some importance in determining the effectiveness of the binding of the compound to the enzyme. The most reactive chemical sites in the estradiol molecule are its two OH groups, located at what might be considered the "ends" of the molecule. These may be involved in both the binding and activating properties of the molecule. It would appear that a molecule must, in addition to having the proper reactive groups, be of the correct size and shape before it can be attached to the enzyme. In contrast, the introduction of unsaturation in ring B, as in equilin and equilenin, appears to have no effect on either the binding or activating properties of the estrogen.

Estriol, and to a much smaller degree, stilbestrol, are slightly effective in activating the enzyme, for they are estrogenic at high concentrations. However, estriol decreases the enzyme activating effect of estradiol when the two are added together. This suggests that estriol competes with estradiol for a binding site on the enzyme but, being much less effective than estradiol in activating the enzyme, has the net effect of decreasing its activity. Estriol was found to be much less effective than estradiol or estrone in stimulating uterine growth in castrate rats (11). Furthermore, when estriol was injected with estrone or estradiol it decreased the growth-promoting effects of these steroids. Hisaw *et al.* postulated that estriol might stimulate the release of ACTH and consequently of adrenocortical steroids, and that the antiestrogenic activity might result from the antagonistic effect of the adrenal corticoids. The results from our experiments would suggest that the antiestrogenic activity of estriol might be a direct effect.

Our analyses of tissue extracts have shown that there are about 200 μg. of estradiol plus estrone per kilogram of fresh placenta (12). This figure agrees very well with the analyses published this year by Diczfalusy and Lindqvist (13). Thus, even when no estradiol is added to the incubation flask, the stilbestrol or other inhibitor added displaces the endogenous estradiol and thus decreases the activity of the enzyme system.

Enzyme inhibitors at the substrate level have been recognized for many years and their mode of action has been explained in this fashion. The present example of a competitive inhibitor of an activator of an enzyme is, however, somewhat different. Its physiologic significance is unclear, for neither stilbestrol nor estradiol-17α is a normal constituent of the body. The interactions of estradiol-17β and estriol might provide the basis for a physiologic "feed-back" system to regulate the activity of the enzyme.

SUMMARY

Stilbestrol, 1, 3 di-*p*-hydroxyphenyl propane (C-3) and estradiol-17α inhibit the activation of placental isocitric dehydrogenase by estradiol-17β. The inhibition by these substances is competitive; higher concentrations of stilbestrol inhibit the reaction more effectively than lower con-

centrations and stilbestrol inhibition is more effective at lower concentrations of estradiol-17β.

Estriol is slightly estrogenic when added alone, but decreases the stimulatory effect of estradiol when added together with it. Ten other compounds tested showed no significant estrogenic or antiestrogenic activity.

The results of these experiments suggest that estrogens and related compounds have two distinct properties: an ability to combine with the isocitric dehydrogenase and an ability to activate the enzyme after combination has occurred. The marked effect of estradiol-17α, stilbestrol and C-3 as competitive inhibitors of estradiol-17β is interpreted as a strong ability to unite with the enzyme but little or no ability to activate it.

The implications of these observations with respect to the molecular size and shape of estrogens and antiestrogens is discussed.

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NOTES AND COMMENTS

MATURATION OF THE INFANTILE TESTES IN RAINBOW TROUT (*SALMO GAIRDNERII*) PRODUCED BY SALMON PITUITARY GONADOTROPHINS ADMINISTERED IN CHOLESTEROL PELLETS[1]

There seems to be almost unanimous agreement among workers in the field that injection of pituitary glands taken from other fish in a suitable state of sexual maturity will cause acceleration of spermatogenesis or oogenesis or both in fish with maturing gonads (1,2). However no such uniformity of effects has been produced in fishes with infantile gonads. Some workers, Azevedo and Canale (3) and Mathews (4) succeeded in inducing spermatogenesis in immature fish with pituitary extracts while others, Ball and Bacon (5), working with different species obtained negative results.

In the present study juvenile fish (rainbow trout) with testes in the undeveloped state, were employed, since such test objects provided a fixed base line to which the results of artificial gonad stimulation could be related. This is lacking in the sexually maturing fish and as a consequence greatly complicates the interpretation of the experimental findings. The gonadotrophic property of the pituitary was concentrated by chemical fractionation (not previously reported in fishes), desiccated and administered quantitatively in the form of cholesterol pellets. The fractions were also injected in suspension for comparison.

MATERIALS AND METHODS

The pituitaries were secured from sexually mature fish, principally chum salmon (*Oncorhynchus keta*), but also from king salmon (*O. tschawytscha*), at the time they were being stripped for eggs or sperm. The glands, taken for the most part immediately after death or not later than 2–3 hours thereafter, were dropped quickly into a Dewar flask containing dry ice and left frozen until they could be lyophilized. Male and female pituitaries were kept separate. Pituitary collections were made over a period of three years.

The lyophilized pituitary tissue was fractionated into three crude protein mixtures. Fraction #1 was extracted at room temperature for 24 hours in a solution of glacial acetic acid and acetone; 16 ml. of acid and 2.5 ml. acetone per gram of tissue. One volume of distilled water was then added. After thorough mixing the suspension was centrifuged, the insolubles being preserved by lyophilization for further study. Acetone was added to the supernatant, with stirring, until heavy turbidity was evident. At this point additional acetone was added cautiously until a flocculent precipitate formed, leaving a clear supernatant. The solids were collected by centrifuging, and washed twice with ethyl ether. The washed precipitate was redissolved in water, enough glacial acetic acid being added to assure solution. After warming at 37° for a few minutes to remove excess ether, the solution was frozen and lyophilized. Yield: approximately 13% of starting weight.

Fraction #2: To the supernatant fluid remaining after the precipitation of Fraction #1 at least one volume of acetone was added. If necessary, further acetone was added to reduce the turbid suspension to a flocculent precipitate. The solids were collected by centrifuging and prepared for lyophilization as with Fraction #1. Yield: approximately 11% of starting weight.

The fractions were made into pellet form by mixing with cholesterol in the desired weight ratio. A small quantity of ethyl ether was added to this mixture. Holding the suspension in a small beaker in the palm of the hand, the whole was stirred with a glass or stainless steel rod under a fume hood. As the ether evaporated care was taken to keep the solids in suspenion by removing them from the beaker sides as residues formed. With reduction in liquid volume a paste formed which, on continued stirring, became a finely divided powder. The desired weight of powder was placed in a die of a simple hand press and the pellet formed by pressure. The diameter of this die was bored to match the internal diameter of a pellet gun.

Rainbow trout varying in age from 6 to 19 months were employed. Since the sex of the

[1] This study was aided by grants from the National Science Foundation and the United States Fish and Wildlife Service.

immature trout cannot be determined by external appearance, preliminary laparotomy was performed in all trout used in the following experiments. Only fish with thread-like testes were selected—a few are sexually mature at an early age. In most experiments one testis was removed beforehand (6).

RESULTS

Preliminary experiments with salmon pituitary homogenates injected intraperitoneally gave equivocal results. It was found that unilateral gonadectomy alone stimulated growth of the remaining testis as did simple laparotomy though to a lesser extent. A solution to this difficulty was provided by the finding that starvation inhibited the testis stimulation of unilateral gonadectomy but did not interfere with gonadotrophin activity.

Accordingly, unilaterally gonadectomized and starved immature rainbow trout were injected intraperitoneally with 1 mg. doses of salmon pituitary fraction #1. Other trout were similarly injected with equivalent doses of fraction #2. (One mg. of fraction #1 is

TABLE 1. GONADOTROPHIC EFFECT OF SALMON PITUITARY FRACTIONS #1 AND #2 ON TESTES OF STARVED UNILATERALLY GONADECTOMIZED RAINBOW TROUT

Observation period 25 days

	Number of trout in group	Age of trout months	Average weight of excised testis, mg.	Total amount of fraction administered, mg.	Remaining testes at autopsy	
					Weight, mg.	% gain in weight (range)
a. Fraction #1 (injected)	5	16–19	14	15	440	3034 (1483–4240)
b. Fraction #2 (injected)	3	16	9	15	30	226 (144–325)
c. Fraction #1 (pellets)	9	6	9	10	92	899 (378–1400)
d. Controls	7	16–19	18	0	23	63 (0–275)

equivalent to 0.56 of an average salmon pituitary and represents approximately 2 per cent of the original wet weight of that gland.) Injections begun on the fifth day after operation were continued daily for 5 days a week over a period of 3 weeks. Marked increase in the weight of the remaining testis occurred in every fish receiving fraction #1 while those injected with fraction #2 showed only a small gain. The uninjected controls exhibited an insignificant change in the size of the testes (Table 1). Microscopical examination of the enlarging testes showed them to have reached the stage of abundant spermatocyte production. The only abnormal feature was the presence of diffuse edema. Other effects of fraction #1 were first a marked generalized pallor, which began to appear within 10–15 minutes and continued to increase for an hour or more, changing to a lemon yellow or sometimes a pale golden tint. The color transformation was more intense after fraction #2 and would last for a matter of days following a single injection. A second effect was the occurrence of exophthalmos which appeared in an hour or two. Thirdly, the thyroid showed marked stimulation. Neither exophthalmos nor thyroid hyperactivity occurred after fraction #2.

Administration of fraction #1 in the form of cholesterol pellets, 5 mg. of fraction #1 and 25 mg. of cholesterol, likewise produced a well marked increase in testis weight within 25 days, though considerably less than that resulting from injection of this fraction even allowing for the smaller dosage used (Table 1c). The pellets were implanted either intraperitoneally or intramuscularly—a specially made trocar-implanter was employed for

TABLE 2. FULL SEXUAL MATURITY PRODUCED IN TROUT WITH INFANTILE TESTES BY INTRAMUSCULAR IMPLANTATION OF MALE OR FEMALE SALMON PITUITARY FRACTION #1 IN CHOLESTEROL PELLETS

Trout fed and observed for 9 weeks

Number of trout in group	Age of trout months	Total amount of fraction #1 administered, mg.	Testes at autopsy	
			average weight, mg.	maturity
1	6	5 ♀*	10,040	+
2	6	10 ♂	5,354	+
				±
1	6	20 ♂	500	±
1	6	20 ♀	2,530	+
Controls				
8	6	0	37	0

* Fraction #1 made from salmon pituitaries of sex indicated.
± = some oozing of milt from spermatic duct or cut surface but non motile spermatozoa.
+ = abundant oozing of milt from spermatic duct and cut surface. Motile spermatozoa.

the latter route. The color change was the same as that following the injection of fraction #1 but there was no accompanying exophthalmos or thyroid stimulation in the pellet-implanted fish and no edema in the developing testes. Thus, except for the color change, the process of gonad development stimulated by fraction #1 pellets resembled that occurring in the normally maturing trout.

In further experiments with pellets of fraction #1 the trout were observed for a period of two months. One such experiment is displayed in Table 2. The testes in 3 of the 5 test trout were fully mature. Milt expressed from these fish before death contained highly motile spermatozoa. The extent of testes growth is shown in Figure 1. Weights of the intact pellets recovered at autopsy revealed an average of 66% absorption of protein as calculated from the weight loss.

FIG. 1. Testes of juvenile trout (right) 2 months after intramuscular implantation of pellet containing 5 mg. of salmon pituitary fraction #1. Oozed milt showing motile spermatozoa. Control testes shown are from a trout of same length and appeared to be similar to those of pellet-implanted trout at beginning of experiment. Reduced to ¾ size.

SUMMARY

A potent gonadotrophic preparation was made from the lyophilized pituitaries of spawning salmon by means of chemical fractionation. Injection into rainbow trout with infantile testes produced marked enlargement of the gonads and active spermatogenesis under conditions of starvation which inhibits spontaneous testicular growth and development. Administration of the gonadotrophin in cholesterol pellets brought about full maturity of the testes of young trout and shedding of motile spermatozoa in a period of two months. The testes of the control trout remained in the immature state.

Acknowledgment

The authors wish to express their appreciation to Mr. Roger E. Burrows, Director of the Salmon Cultural Laboratory, U. S. Fish and Wildlife Service of Entiat, Washington, for his generous cooperation in providing most of the salmon pituitaries used in this study.

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EFFECT OF ALDOSTERONE ON RENAL EXCRETION OF INTRAVENOUSLY ADMINISTERED SALINE[1]

Isotonic saline administered intravenously to rats causes a considerable diuresis and increases the rate of urinary sodium loss (1, 2, 3, 4). This increased rate of sodium loss, due mainly to decreased renal tubular sodium reabsorption (2), cannot entirely be prevented by giving either desoxycorticosterone acetate (DCA) or glycoside (DCG). O'Connor (5) has reported similar findings in dogs. The action of aldosterone, given either intravenously or intramuscularly, has now been investigated.

Adult, male albino rats (Glaxo strain) of about 250 gm. body weight were used in all experiments. A polythene cannula had been implanted in the external jugular vein two of three days before the infusion (6). On the day of the experiment, without using any anaesthetic, isotonic Krebs' (bicarbonate) solution was infused through the cannula at 0.150 ml./min. and urine was collected from a polythene funnel, draining into a graduated centrifuge tube. During the first two hours of the experiment urine was

Received October 12, 1956.

[1] This work was done during the tenure of an I.C.I. fellowship.

collected at intervals of thirty minutes and thereafter, hourly. Endogenous creatinine clearance was used as an estimate of glomerular filtration rate (GFR) and the methods of blood collection, storage of specimens and chemical analyses were similar to those described previously (2). The rates of excretion of water and sodium, expressed as percentages of the rates of filtration, were calculated from the creatinine and sodium urine concentration ratios (2). These two values, the "tubular rejection fractions" (TRF_W and TRF_{Na}) are not subject to collection errors since the rate of urine flow does not enter into the calculation.

The control group consisted of 12 animals; the two "aldosterone groups" of six each. Intramuscular aldosterone was given as an oily solution containing 10 μg. two hours before starting the infusions. Intravenous aldosterone, 10 μg. was given as an emulsion of the oily solution in saline thirty minutes before the infusion.

The Figures show smoothed curves based upon the mean values of variables, at each time interval.

From Figures 1 and 2 it is apparent that aldosterone administered, by either route, can reduce the sodium loss and increase its reabsorption in the renal tubules. It is possible that the reduced water loss may be secondary to this effect. Aldosterone also decreases the ratio of sodium to potassium in the urine. There is no significant action on GFR. Nevertheless, the increased sodium excretion in response to isotonic infusion is not abolished by aldosterone, corresponding with our findings with DCA and DCG.

Singer and Stack-Dunne (7, 8) indicate that a single rat adrenal may produce the equivalent of 45 μg. DCA/Kg. bodyweight/hr., i.e., for a 250 gm. rat, the equivalent of

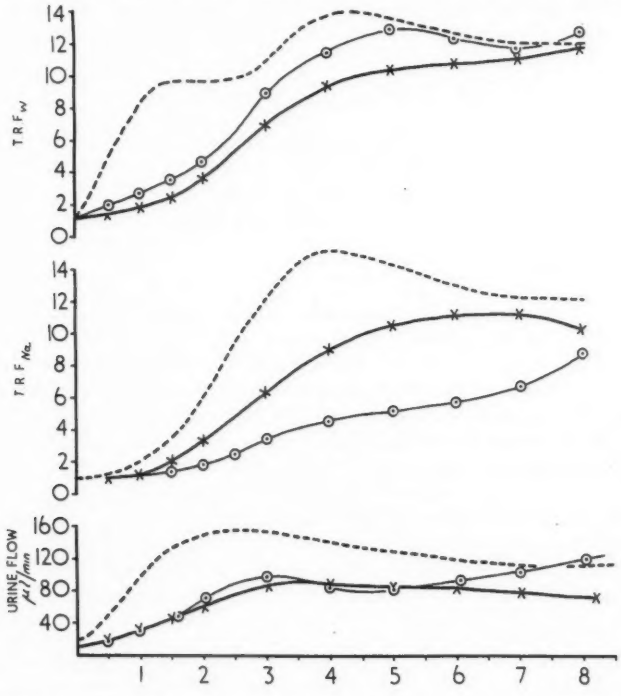

FIG. 1: Values for TRF_W (top). TRF_{Na} (center) and rates of urine flow in μl. per minute (bottom).

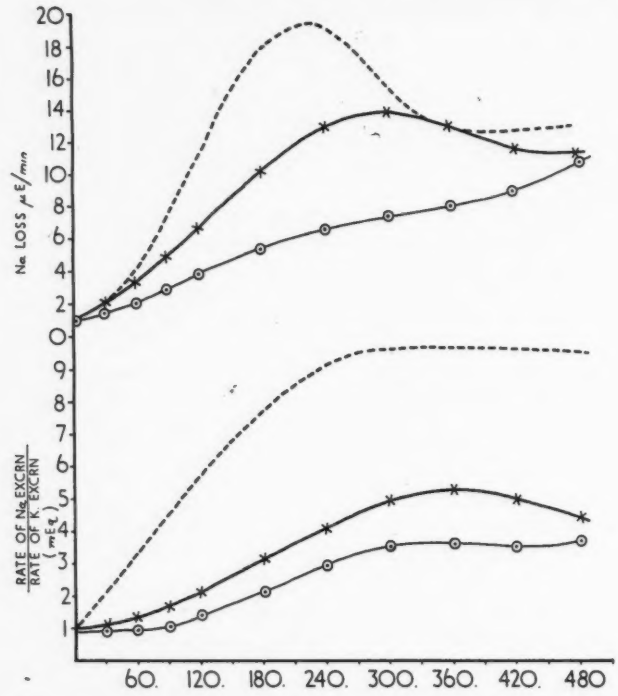

FIG. 2. Values for the rate of urinary sodium excretion in μEq. per minute and for the ratio of the excretion rates of sodium and potassium. In both figures the broken line (- - - -) represents data from the control group, open cicles (○ - - - - ○) represent the group given aldosterone intramuscularly and crosses (× - - - - ×), the group given aldosterone intravenously.

22.5 μg. DCA/hr. Assuming the ratio of aldosterone activity to that of DCA to be 30:1 (cf. 9) the above values suggest that the aldosterone output of a 250 gm. rat would be about 0.75 μg./hr. Apart from the control series, all our animals were given at least 10 μg. aldosterone, which represents not less than a ten hour supply. Granted that the aldosterone supply was adequate, it would seem that the increase of sodium excretion after isotonic infusion cannot be attributed solely to decreased aldosterone production by the animals' own adrenals.

Acknowledgment

The author is greatly indebted to Dr. F. Gross, CIBA Aktiengesellschaft, Basel, Switzerland, for the aldosterone used in this work.

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THYROIDAL FUNCTION IN FETAL AND PREGNANT SHEEP GIVEN CHRONIC LOW LEVEL DOSAGES OF I^{131} [1]

Little is yet known concerning the development in the mammalian fetal thyroid gland of the ability to synthesize thyroid hormone. The small size of the gland at the time function begins in the usual small laboratory mammals makes it difficult to obtain sufficient material for analysis of the various iodine compounds which could be present. Recently, Barnes and others (1) reported that significant accumulation of radioiodine by thyroids of fetal sheep begins at about the 50th of 145 days of gestation. They showed, furthermore, that the fetal thyroidal concentration increases exponentially after this (2).

METHODS

Samples of frozen maternal and fetal thyroid tissue from the experiment of Barnes *et al.* were supplied to the Department of Zoology, Columbia University for chromatographic analysis of the radioactive iodine labelled compounds present at different stages of pregnancy. The five adult ewes used in the work were maintained on a diet low in iodine and fed 5.0 microcuries of I^{131} daily beginning over three years before pregnancy. These animals were sacrificed on days 70, 85, 100, 115, or 130 of pregnancy. In addition, one ewe was fed only a single dose of 320 microcuries of I^{131} 24 hours before sacrifice.

The thawed thyroid tissue was ground in a glass homogenizing vessel and suspended in physiological saline solution. An aliquot of this suspension was used for measurement of total radioactivity. The remainder was digested with trypsin, followed by papain, and analyzed by filter paper chromatography, using techniques previously described, (3).

RESULTS

The concentration of isotopic iodine in the fetal thyroid increased from about one-hundredth that of the maternal thyroid tissue to approximate equality (Table 1).

The maternal thyroids in all stages of pregnancy contained some radioactive thyroxine in addition to mono- and diiodotyrosine. The youngest fetal sheep thyroid examined (70 days gestation) contained a barely detectable amount of thyroxine. Less than 5% of the radioiodine was in this form. Except for the 115-day-old fetus, radioactive thyroxine was found in the older embryo thyroids in easily detectable amounts. Radioactive triiodothyronine was found in both maternal and fetal thyroids, but irregularly, and according to no recognizable pattern of distribution. However, it may be said that

Received October 15, 1956.

[1] This paper is based on work which was partially performed under Contract No. W-31-109-Eng-52 for the Atomic Energy Commission. Part of the research was supported by a grant from the National Science Foundation.

TABLE 1. CHROMATOGRAPHIC RESULTS AND RATIO OF I^{131} IN MATERNAL AND FETAL THYROID GLAND AT VARIOUS PERIODS OF GESTATION

Ewe	Fetal Age (days)	Fetuses	Treatment	I^{131}/mg. thyroid (fetus:dam ratio)	Radioactive amino acids[1] in thyroid hydrolysates	
					Mother	Fetus
A	70	1	Daily 5 μc. I^{131}	0.013	MIT, DIT Tx, TITn	MIT, DIT Tx-trace
B	85	2	Daily 5 μc. I^{131}	0.060[2]	MIT, DIT Tx, TITn-trace	MIT, DIT Tx
C	100	2	Daily 5 μc. I^{131}	0.40[2]	MIT, DIT (Tx-TITn)[3]	MIT, DIT (Tx-TITn)[3]
D	115	1	Daily 5 μc. I^{131}	1.10	MIT, DIT Tx, TITn	MIT, DIT Tx, TITn
E	130	2	Daily 5 μc. I^{131}	0.86[2]	MIT, DIT Tx, TITn	MIT, DIT Tx, TITn
F	121	2	Single dose 320 μc. I^{131} on day 120	0.73[2]	MIT, DIT Tx, TITn	MIT, DIT Tx, TITn

[1] Key to symbols used: MIT —monoiodotyrosine
DIT —diiodotyrosine
Tx —thyroxine
TITn—triiodothyronine

[2] Average of two fetuses.

[3] In this series of chromatograms it was not possible to distinguish with certainty between thyroxine and triiodothyronine.

triiodothyronine was absent from thyroids of the youngest fetuses, but it was easily demonstrable in the oldest fetuses. All thyroids contained iodide, monoiodotyrosine, and diiodotyrosine.

SUMMARY

A more than hundred-fold increase in tracer iodine concentrating ability occurred between gestation days 70 and 130 in thyroids of fetal sheep whose mothers are fed 5 μc. of radioiodine daily. By 115 days of gestation, the maternal and fetal thyroidal iodine concentrations are about equal.

Even while fetal thyroidal iodine concentrating ability is quite weak, the ability to form iodotyrosines, triiodothyronine, and thyroxine exists. Thus, the mechanism and capacity for thyroxine synthesis are differentiated in sheep long before the adult rate of hormone synthesis is attained.

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THE EFFECT OF HYPOTHALAMIC LESIONS UPON THE THYROID RESPONSE TO PARTIAL THYROIDECTOMY[1]

In the studies of Greer (1, 2) and Bogdanove and Halmi (3) it has been demonstrated that specific hypothalamic lesions prevent the usual goitrogenic effect of propylthiouracil feeding in rats while they do not prevent the increase in iodide-trapping ability of the thyroid gland which follows treatment with this goitrogen. A similar dissociation was observed in propylthiouracil fed hypophysectomized mice bearing intra-ocular pituitary transplants (4). Greer's suggestion that this constitutes evidence for the existence of two kinds of thyroid-active pituitary hormones has been criticized by Halmi and Spirtos (5) and by VanderLaan and Caplan (6) on the basis of their finding that the thyroid iodide trap varies independently of the size or morphology of the thyroid gland when TSH treated hypophysectomized animals are given propylthiouracil (5, 6) or maintained on a low iodine diet (6).

In order to avoid the ambiguities introduced by propylthiouracil feeding this problem has been studied by observing the changes in the thyroid iodide trap and in thyroid size which follow three-quarter thyroidectomy in rats with anterior hypothalamic lesions. The precise localization of the lesions and data on other changes in iodine metabolism will be reported in a later communication. The results presented below indicate that even in the absence of propylthiouracil, certain hypothalamic lesions bring about a dissociation between the hyperplasia and the increase in iodide trapping ability which follow partial thyroidectomy in normal animals.

METHODS

Male rats of the Sprague-Dawley strain were used in these studies, were housed in a controlled temperature room at 21 ± 1° C and were given Purina Laboratory Chow and tap water *ad lib.* Hypothalamic lesions were produced in a series of 42 animals, utilizing the Krieg stereotaxic instrument. Bilateral lesions were placed between 1 mm. posterior and 2 mm. anterior to bregma, 0.5 to 1 mm. lateral to the sagittal suture, and 0.5 mm. from the floor of the skull. Sixteen control animals were similarly anaesthetized, the skulls trephined, and electrodes inserted, but no current was passed through the electrodes. Four to five weeks after the production of lesions, the animals were subjected to three-quarter thyroidectomy under ether anaesthesia. The weight of the thyroid remnant was estimated by subtracting from the weight of the removed left lobe the weight of the removed portion of the right lobe of the thyroid gland. At the time of the partial thyroidectomy, the thyroid:serum iodide ratio was determined, utilizing a modification of the method of VanderLaan and Greer (7). This was done by injecting 6 mg. of PTU suspension in 0.01 N NaOH intra-peritoneally followed one hour later by the subcutaneous injection of 5 μc. of I^{131}. The operation was performed one hour after the radioiodine injection, and immediately after removel of the thyroid gland a blood specimen was collected from the tail vein. Radioactivity in the left lobe of the thyroid gland and in 0.1 ml. of serum was determined in a well-scintillation counter, and these data used to calculate the T/S ratio. Four to five weeks after the partial thyroidectomy, the remnant of the thyroid was removed and weighed, a specimen of tail blood obtained and the T/S ratio determined as at the initial operation.

Received October 19, 1956.

[1] This work was supported in part by United States Public Health Service Grant B-834 and by a grant from the Commonwealth Fund.

RESULTS

A total of 31 rats with lesions and 12 control animals survived all three operative procedures. One of the control animals failed to gain weight after partial thyroidectomy, and was discarded from the series. Of the 31 rats with lesions (Table 1) 10 animals showed no compensatory hypertrophy, 10 animals showed partial compensatory hypertrophy, and 11 animals showed hypertrophy of a degree equal to or greater than the lowest value in the control group. By contrast the control animals all showed hypertrophy of the remnant which averaged 163%.

Partial thyroidectomy was followed by an increase in T/S ratio in all groups, whether or not compensatory hypertrophy of the remnant had occurred. On the other hand, some effect of the hypothalamic lesions on the iodide trapping capacity of the thyroid gland was indicated, since the average T/S ratio in operated animals was significantly less than in the control group ($P < .005$). The observed effect of the hypothalamic lesions on compensatory hypertrophy was not a consequence of inanition, as is indicated by the

TABLE 1. THE EFFECT OF ANTERIOR HYPOTHALAMIC LESIONS UPON THE CHANGES IN THYROID WEIGHT AND THYROID/SERUM IODIDE RATIO WHICH FOLLOW THE REMOVAL OF THREE-QUARTERS OF THE THYROID GLAND IN THE RAT

Group	No. of animals	Body weight (gm.)			Thyroid weight (mg.)		Thyroid:Serum iodide ratio	
		Initial	After* lesion	At death	Remnant left	Remnant found	Initial	Final
Control	11	183	271	350	4.0±0.55	10.6±1.05	34.3±4.5	61.2± 8.5
No Regeneration	10	187	270	322	4.0±0.52	2.4±0.44	26.4±3.8	50.2± 9.4
Partial Regeneration	10	178	230	311	4.8±0.48	7.4±0.74	15.0±2.4	54.0±12.9
Full Regeneration	11	180	265	365	3.0±0.40	9.3±1.18	18.6±3.3	49.3± 6.8

* At time of partial thyroidectomy.
S.E. given in table.

fact that weight gain was noted in all four groups. The smaller weight gain in animals that showed no or reduced thyroid regeneration is probably a consequence of reduced thyroid function, rather than a cause of it. This is suggested by the fact that in the interval between the production of the lesion, and the partial thyroidectomy, weight gain in the group with no regeneration was comparable to that occurring in the control group.

DISCUSSION

These studies confirm Greer's findings (1, 2) that certain hypothalamic lesions prevent the thyroid hypertrophy that usually follows lowering of the blood thyroid hormone level. These lesions induce some decrease in the iodide-trapping ability of the thyroid gland but do not prevent the increase in iodide-trapping ability which follows partial thyroidectomy. The dissociation between growth and T/S ratio changes seen in rats with hypothalamic lesions is produced not only by propylthiouracil feeding, but may also be brought about by partial thyroidectomy.

These observations are in harmony with but do not prove Greer's hypothesis that there are two kinds of TSH, one under nervous control which influences growth of the thyroid gland and the other independent of the nervous system which influences iodide metabolism. The critical issue in the acceptance of this hypothesis is concerned with the extent to which T/S ratio changes are a more sensitive indicator of TSH effect than are the morphological changes in the thyroid gland.

There are good data to support the view that there is no greater sensitivity of the iodide trap than of the morphological response to TSH. This has been demonstrated by administering graded doses of propylthiouracil or graded doses of thyroxine to rats on a fixed intake of propylthiouracil (2). In both experiments changes in T/S ratio were

parallel to changes in thyroid weight. Halmi and Spirtos (5) have shown furthermore that both T/S ratio and thyroid cell height increase progressively over the dosage range of 0.05 to 0.3 USP units of TSH per day.

Under certain conditions, however, the response of the thyroid iodide trap appears to be a more sensitive indicator of TSH activity than does the growth response. This is noted when TSH is administered with propylthiouracil (5, 6) or to animals on a low iodine diet (6). In the present study the rats were not fed a low iodine diet, nor were they given propylthiouracil, so that these causes of dissociation cannot be invoked here. Apparent greater sensitivity of the iodide trap to TSH than of the morphological response has been observed following hypophysectomy in propylthiouracil treated animals (7) but in this instance hyperplastic changes were present when iodide trapping was at a minimum, a situation which is the reverse of that seen in rats with hypothalamic lesions. A single injection of TSH in hypophysectomized rats (8) may also cause an increase in T/S ratio without any detectable morphological response. Under these special conditions the apparent greater sensitivity of the iodide trap to TSH may simply reflect a different time course of response of the varied aspects of thyroid function. The circumstances described above in which dissociation of effect of TSH are noted are probably not applicable to the present observations of the long term effects of partial thyroidectomy but they do demonstrate that thyroid size and iodide trapping ability may at times vary independently.

The findings in the present study like those of Greer are most readily explained by postulating the existence of two kinds of TSH. In view of the present uncertainty as to the significance of changes in T/S ratio however, further experiments will be required before the existence of two kinds of TSH can be proved.

CONCLUSIONS

Three-quarter thyroidectomy is followed by an increase in iodide trapping capacity of the thyroid remnant as well as by compensatory hypertrophy. Lesions placed in the anterior hypothalamus reduce the iodide-trapping capacity of the thyroid gland, but do not prevent the increase that follows partial thyroidectomy. On the other hand the usual hypertrophic response of the thyroid remnant may be abolished or reduced by such lesions.

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THE IMPORTANCE OF CORTISONE IN PROMOTING ESTROGEN-WITHDRAWAL BLEEDING IN THE HYPOPHYSECTOMIZED BABOON

Estrogen-withdrawal bleeding can be induced in hypophysectomized female baboons pretreated with adrenocorticotrophic hormone (ACTH) and thyroxine (1). Such bleeding fails to occur after a course of estrogen alone or of estrogen in conjunction with ACTH.

The desirability of ascertaining whether withdrawal bleeding would occur following a course of treatment with estrogen and cortisone became patent. Accordingly, an adult female baboon (B 359) was hypophysectomized. Forty-eight days after the operation, a course of treatment with estrogen (0.1 mg. estradiol benzoate daily) was instituted and continued for 14 days. The sex skin reacted in the way previously described for hypophysectomized baboons, and, as in the other similarly-treated hypophysectomized baboons, neither macroscopic nor microscopic bleeding occurred during a period of 21 days following the withdrawal of estrogen treatment.

After a further interval of three weeks, the baboon received daily injections of cortisone (1 mg./kg. body weight) for 44 days, and thereafter 0.1 mg. estradiol benzoate in addition to the cortisone for 14 days. Estrogen treatment was then withdrawn but cortisone injections continued. The sex skin underwent involution and external vaginal bleeding occurred on the 10th day after the last injection of estrogen. Bleeding continued for 3 days.

Six weeks later, the baboon was again challenged with estrogen without the occurrence of withdrawal bleeding.

From this experiment, it can be concluded 1) that cortisone, in the concentrations used, can create the conditions necessary for estrogen withdrawal bleeding in an hypophysectomized baboon and 2) that estrogen-withdrawal bleeding can occur in the absence of added thyroxine, provided sufficient cortisone is available. It is also evident that the kind and amount of ACTH used in previous experiments (1) was not able to stimulate the adrenal of the baboon to produce cortisone in amounts equivalent to those supplied by injection in the experiment in B 359. In this connection, it should be noted that ACTH was not as efficient as cortisone in improving the capacity of hypophysectomized baboons to excrete a test load of water (2).

Finally, it is apparent that a functionally active adrenal cortex appears to be an essential component of the estrogen-withdrawal bleeding mechanism in hypophysectomized baboons.

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Received October 15, 1956.

FAILURE TO DETECT SQUAMOUS METAPLASIA IN EXTRA-GENITAL ORGANS OF THE FEMALE RAT FOLLOWING PROLONGED ESTROGEN ADMINISTRATION

It is known that in rats either prolonged estrogen administration or the giving of a diet deficient in vitamin A may be followed by the appearance of squamous epithelial metaplasia. In vitamin A deficient rats this metaplasia is seen in organs of the urogenital system and in other organs too (1). Following estrogen administration it has been observed in various organs of the urogenital system in different animals (2); in the female rat it has been found to take place in the uterus (3, 4).

The present investigation was undertaken to determine whether estrogen administration in female rats is followed by the occurrence of squamous metaplasia in organs outside the urogenital system as in the case of vitamin A deficient rats.

MATERIALS AND METHODS

Pellets of estradiol benzoate of known weight were implanted subcutaneously into the left inguinal region of 29 to 31-day old female white rats. The animals were killed after varying intervals of time (28–192 days), having been kept on a diet consisting of a mixed chick feed, supplemented twice weekly with germinating wheat moistened with fish liver oil. To determine the amount of estrogen absorbed the pellets were dissected out and, after drying, re-weighed.

Histological technique consisted of fixation in Bouin's solution, embedding in paraffin and staining with hematoxylin and eosin. Random sections from different parts of the embedded tissue were mounted and examined; the number of sections of each organ examined varied from 4 to 12. The number of animals used is shown in Table 1.

A preliminary experiment had been conducted earlier in order to determine whether vitamin A deficiency in the strain of rats being used here produces squamous metaplasia. A group of ten rats, 30–32 days old at the start of the experiment, were kept on a diet consisting essentially of the U.S.P. Vitamin A test diet,[1] very slightly modified, for periods varying from 67–154 days.

RESULTS

The amount of estrogen absorbed varied from 2.2 to 11.7 mg., being generally greater in animals in whom the experiment had lasted longer. In some instances it was found that the whole pellet had been absorbed.

In the estrogen-treated animals, squamous metaplasia was not detected in any of the extra-genital organs examined (Table 1). That estrogen was in fact being absorbed and was acting in these animals is evidenced by the fact that squamous metaplasia was observed in the uteri of five animals; it is also attested to by the presence, in most of the rats, of other manifestations of prolonged estrogen administration, such as macroscopically-evident pus in the uterine cornua, hair loss and failure to gain in weight (5, 6).

In the vitamin A deficient animals squamous metaplasia was found in various organs, as shown in Table 1.

DISCUSSION

The failure to detect squamous metaplasia, following prolonged estrogen administration, in organs outside the urogenital system is in accord with the findings of McCullough and Dallforth (7) on the trachea and thyroid and of Hume *et al.* (8) on the trachea and intrapulmonary bronchi. The latter authors make a passing reference to having found squamous metaplasia in the thyroid of "a few" male rats after estrogen administration but they provide no details.

Cramer (9) has expressed the opinion that estrogen exerts its metaplastic effect on

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Received November 4, 1956.

[1] The United States Pharmacopoeia. Twelfth Revision (U.S.P. XII). Mack Printing Company, Easton, Pa. 1942.

TABLE 1. RESULTS OF ESTROGEN ADMINISTRATION AND OF VITAMIN A DEFICIENCY EXPERIMENTS

Organ	Implantation of estrogen pellet		Vitmain A deficient diet	
	No. of animals in which organ examined	No. in which squamous metaplasia seen	No. of animals in which organ examined	No. in which squamous metaplasia seen
Parotid gland	15	0	10	8
Submaxillary gland	15	0	9 (1)	6
Major sublingual gland	14 (2)	0	8 (1)	2
Thyroid gland	15 (1)	0	8	8
Trachea (region of bifurcation)	15	0	9	5
Lung (right upper lobe)	15	0	9	5
Uterus (cornua)	15	5	8	2
Harderian gland (left)	12	0	—	—
Exorbital lacrymal gland	16	0	10 (1)	0
Parathyroid glands	14 (5)	0	8 (2)	0
Stomach (pyloric region)	15	0	9	0
Duodenum	14	0	8	0
Pancreas	14	0	8	0
Jejunum	15	0	5	0
Caecum	14	0	7	0
Rectum	15	0	7	0

In the case of bilateral organs, those of both sides were examined except where otherwise stated. Also, in that number of instances indicated in parenthesis the organ of one side only was examined.

the epithelium of the uterus by producing a local (tissue) vitamin A deficiency. He found that high doses of vitamin A prevent the appearance of squamous metaplasia in the uterus of estrogen-treated rats and similar results were obtained by McCullough and Dallforth (7).

In this connection Fell and Mellanby's (10) studies of the differentiation of chick ectoderm in tissue culture are also of significance. They found that whereas, in a normal medium, the ectoderm differentiated into a keratinizing, stratified squamous epithelium, in a high vitamin A medium this type of development was inhibited and the ectoderm differentiated into mucus-secreting, often ciliated epithelium.

It is possible that the negative results of the present investigation signify that prolonged estrogen administration in the rat is incapable of inducing a local vitamin A deficiency in organs outside the urogenital system.

SUMMARY

The examination of various extra-genital organs of female rats following prolonged estrogen administration failed to reveal the presence of squamous epithelial metaplasia such as occurs in some of these organs in rats kept on a vitamin A deficient diet.

Acknowledgments

I am indebted to Professor K. Guggenheim for helpful advice in connection with the vitamin A deficiency experiment and to Miss T. Daskal and Mr. I. Hayoun for extensive technical assistance.

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DECREASED SENSITIVITY OF THE EOSINOPHIL DEPLETION TEST IN THE RAT EXPOSED TO A LOW AMBIENT TEMPERATURE[1]

An increased utilization of adrenocortical hormones by the peripheral tissue has been suggested as an explanation of the increased secretion of ACTH following application of a stressful stimulus (1). If such were the case it might be possible to obtain a decreased sensitivity to cortisone following application of a stressor.

In this experiment, the eosinopenic response to a standardized dose of cortisone was measured in splenectomized-adrenalectomized rats kept at room temperature (22–24° C) and in a cold room (3–5° C).

MATERIAL AND METHODS

Mature, male rats of the Harvard-Wistar strain, weighing 250 to 350 gm., were used in these experiments. The animals were first splenectomized and then adrenalectomized several days following the initial operation. A pellet of desoxycorticosterone acetate weighing from 30 to 60 mg. was implanted subcutaneously in each rat at the time of adrenalectomy. The animals were then given one week for post operative recovery prior to experimentation and maintained at a room temperature of 22–24° C during this period. Food and water were supplied *ad libitum*. The rats were then divided into three groups. Two groups were maintained at room temperature for the duration of the experiment and the third group was placed in a cold room at a temperature of 3 to 5° C for 7 days.

The cortisone acetate was suspended in saline with "Tween 80" and injected subcutaneously in a volume of 0.1 mg. Blood samples for eosinophil counts were obtained by cardiac puncture and diluted with the fluid described by Pilot (2). Eosinophil counts were obtained at the time of injection of the hormone and 6 hours later (3). All values are expressed as the per cent difference between the initial and the 6-hour count.

DATA AND DISCUSSION

It may be seen from the data in Table 1 that 20 μg. of cortisone acetate produced an eosinopenia of 34% in rats kept at 22–24° C but the drop was not significantly different from the pre-injection level. However the eosinophil drop of 65% obtained with 40 μg. of cortisone acetate was significant at the 0.05 level. No eosinopenia was obtained in a third group of rats injected with 40 μg. of cortisone acetate and kept at an ambient temperature of 3–5° C for 7 days prior to the injection of the hormone. Thus a dose of cortisone acetate that was adequate for an eosinopenic response in the rat kept at room temperature was ineffectual in the rat kept in the cold.

Received November 8, 1956.

[1] This work was aided by a grant (2-22806) from the National Cancer Institute of the NIH, Public Health Service.

TABLE 1. THE EFFECT OF EXPOSURE TO COLD ON THE EOSINOPHIL DEPLETION TEST IN THE SPLENECTOMIZED-ADRENALECTOMIZED RAT

No. of rats	Cortisone acetate, μg.	Temperature, ° C	% decrease in eosinophils	S.D.	Significant* drop at the 0.05 level
9	20	22–24	−34	±37	−
6	40	22–24	−65	± 9	+
7	40	3–5	− 4	±44	−

* The Duncan-Bonnor test for significance at the 0.05 level was used.

These results demonstrate that the eosinophils of the rats exposed to a low ambient temperature become relatively refractory to cortisone but do not give any explanation for the mechanism involved in the development of the resistance to the hormone. Nevertheless, it is possible to explain this data on the basis of the concept of increased peripheral utilization of the hormone in the rat following exposure to cold. Even here, however, no general conclusion can be reached until some of the contradictory results are explained. Cowie *et al.* (4) reported either no eosinopenia or a greater degree of eosinopenia in the adrenalectomized dog following hydrocortisone and trauma. Ulrich and Long (5) concluded, from experiments on the loss of radio-active adrenal corticoids from the blood of stressed rats, that their data failed to demonstrate increased peripheral utilization. However they also indicated that more elegant experiments will have to be devised to settle this question.

Part of the contradiction between these results and that of the above may be due to the duration and the type of stress. In the present experiment the animals were exposed to a lowered temperature for 7 days whereas Cowie *et al.* (4) used a surgical stress. It is of interest to note that prolonged exposure to cold has been shown to result in a recovery from the initial eosinopenia even though the stress was maintained (6).

SUMMARY

The eosinopenic response to a standardized dose of cortisone acetate was determined in the splenectomized-adrenalectomized rat maintained at a room temperature of 22–24° C and at a temperature of 3–5° C.

The minimal effective dose of cortisone acetate necessary to produce a significant drop in the eosinophils of the blood of the rat was shown to be 40 μgs. at room temperature. This concentration failed to produce a significant eosinopenia in the rat when exposed to a temperature of 3–5° C for 7 days.

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AUTOTRANSPLANTATION OF THE ADRENAL OF THE RAT TO THE PORTAL CIRCULATION: INDUCED ADRENAL HYPERTROPHY AND ITS PREVENTION BY OOPHORECTOMY[1]

Previous studies have demonstrated that autografts of the adrenal implanted in the portal venous bed remain viable and function, as shown by maturation of the rats, continued sexual activity, and the ability to withstand the stress of hypoglycemia better than adrenalectomized controls (1–4). In the course of the above experiments, it was noted that the adrenal autografts appeared larger than control glands. Hence the present study was undertaken to study this phenomenon.

This report will demonstrate that extraordinary adrenal hypertrophy does occur when an adrenal gland is autotransplanted into the portal bed of the adrenalectomized female rat, and that the hypertrophy is prevented by oophorectomy.

METHODS

Experiment 1. Female rats of the Long-Evans strain, which weighed approximately 150 gm. were unilaterally adrenalectomized and divided into four groups of twelve animals each, which were treated as follows: "Group A" served as controls, in "Group B" the remaining adrenal was transplanted to the systemic circulation (i.e. the mesentery of the uterus), in "Group C" the remaining adrenal was transplanted to the portal circulation (i.e. the greater curvature of the stomach adjacent to the splenic veins, rather than directly to the spleen where adhesions were more likely to occur), "Group D" was treated the same as Group C in addition to the parenteral administration of cortisone, 1 mg. daily for 5 days of the week.

Experiment 2. Four identical groups of female rats were prepared and in addition to the above conditions, all of them were castrated at the original operation.

Following surgery, all of the animals in Experiments 1 and 2 were maintained on normal saline instead of tap water, for a period of three weeks. The rats were weighed and autopsies performed after 150 days, except in Experiment 1, Group C, where some autopsies were performed after 50 days. At autopsy, the adrenal gland was carefully freed of adherent tissues, blotted and weighed. If adrenal remnants or accessory tissue were found, the animals were not included in the series.

Twenty normal adrenal glands, and 13 enlarged intraportal adrenal glands were dried to constant weight to calculate and compare the percentage of water in both types of glands. All the rest of the glands were used for histological examination.

RESULTS

Histological Examination

Fifty days after transplantation the adrenals in the wall of the stomach (Experiment 1, Group C) are sharply delimited by a fine fibrous capsule. A thin layer of cells resembling the zona glomerulosa rests on the fibrous capsule. The remainder of the gland is composed of cords of cells patterned after the zona fasciculta. In the central part, there is slight distortion of the regular cords of cells. Most of the cells are polyhedral, fairly uniform in size and shape, with a round nucleus and moderately vacuolated granular cytoplasm. At 150 days after transplantation, there is a distinct increase in septal bands of fibrous tissue and often there is a suggestion of lobulation. In several instances this is accentuated to form an isolated nodular mass, simulating what has been described in the human female after similar adrenal transplantation (5). In many glands there are clusters of cells in the cords of the zona fasciculata which have a condensed homogeneous, eosinophilic cytoplasm, and differ from the adjacent cells with a finely granular foamy cytoplasm. In none of the sections at 50 or 150 days is adrenal medullary tissue seen. The pituitaries in both of these groups are not unusually different in size and weight and routine sections disclose no alteration in cellular pattern.

In the castrated animals (Experiment 2), the pituitaries show the usual castration

Received November 13, 1956.

[1] This work was supported by NIH Grant CY-2079 (C3) Path. and the Florence Hellman Dinkelspiel Memorial Fund for Cancer Research, Mount Zion Hospital.

TABLE 1. CHANGE IN ADRENAL WEIGHT 150 DAYS AFTER RIGHT UNILATERAL ADRENALECTOMY IN FEMALE RATS

Type of experiment	Mean weight of adrenal expressed as Mg./100 gm. of rat					
	No. of rats	Initial	Final	Increment	S.D. of increment	P value*
		(Right adrenal)	(Left adrenal)			
Group A. Left adrenal in situ	10	12.2	21.7	9.5	3.7	
Group B. Left adrenal in systemic circulation	11	12.3	20.5	8.2	11.0	>0.7
Group C. Left adrenal in portal circulation	12	12.8	32.3	19.5	9.7	<0.01
Group D. Left adrenal in portal circulation, parenteral cortisone 5 mg./week	10	14.1	28.0	13.9	13.0	>0.3

* The P value is the probability of obtaining by chance the weight increment when compared with group A.

changes. The transplanted adrenals show a moderate increase in fibrous septal tissue and rare lobulation of cells. However, as in the noncastrate groups no medullary tissue is present.

From Table 1, it is seen that the weight increment that occurs from intraportal transplantation of the adrenal gland is approximately twice as great as the compensatory hypertrophy of the remaining adrenal after unilateral adrenalectomy or transplantation to the systemic circulation.

The enlargement is evident as early as 50 days since 15 animals in Experiment 1, Group C, examined after 50 days, showed a mean adrenal increment of 18.8 mg. per 100 gm. of rat with S.D. = 13.8 and P value <.02.

The enlargement was not due to the accumulation of fluid since the percentage loss in weight after dehydration of 20 normal adrenal glands was 69.5% (S.D. = 0.9) whereas 13 intraportal adrenal glands similarly dehydrated lost 71.4% (S.D. = 0.6).

Examination of the data in Table 2 shows no statistical validity of weight change in any of the groups. Thus the extensive adrenal hypertrophy that occurs from intraportal transplantation is prevented by castration. Moreover even the moderate compensatory hypertrophy of the remaining adrenal gland following unilateral adrenalectomy, with

TABLE 2. CHANGE IN ADRENAL WEIGHT 150 DAYS AFTER RIGHT ADRENALECTOMY AND OOPHORECTOMY IN FEMALE RATS

Type of experiment	Mean weight of adrenal expressed as Mg./100 gm. of rat				
	No. of rats	Initial	Final	Change in weight	S.D. of change in weight
		(Right adrenal)	(Left adrenal)		
Group A. Left adrenal in situ	11	13.0	10.6	−2.4	4.5
Group B. Left adrenal in systemic circulation	11	13.0	9.6	−3.4	2.5
Group C. Left adrenal in portal circulation	12	13.9	14.4	+0.5	5.5
Group D. Left adrenal in portal circulation, parenteral cortisone 5 mg./week	9	13.5	12.8	−0.7	6.1

or without transplantation to the systemic circulation, is eliminated after oophorectomy. In Groups A, B, and D, the final mean adrenal weight is less than the initial weight. At present, there is no explanation for these findings. It is of interest that while the adrenal hypertrophy is eliminated there still remains on histologic examination some prominence of the fibrous septal strands.

From the data presented, the effect of cortisone on the adrenal hypertrophy or on the prevention of hypertrophy by oophorectomy, is negligible.

Further studies on this induced adrenal hypertrophy are in progress.

SUMMARY

An extraordinary hypertrophy is induced in the adrenal gland implanted in the portal venous bed of adrenalectomized female rats, and is prevented by oophorectomy.

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THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

Joint Sessions

The Endocrine Society will hold a joint session with the American Goiter Association on Thursday morning, May 30, 1957, and a joint session with the American Diabetes Association Saturday morning, June 1, 1957. Both of these joint sessions will be held in the meeting room of the Endocrine Society in the hotel New Yorker.

1957 LAURENTIAN HORMONE CONFERENCE

The 1957 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 1–6, and investigators interested in attending this conference should make applications to the Committee on Arrangements of The Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 10, 1957. A conference rate of $12.00 per person per day is extended to all invited participants. Attendance is limited to invitations issued by the Committee on Arrangements.

THE AMERICAN GOITER ASSOCIATION

The American Goiter Association will hold its 1957 Meeting in the Hotel Statler, New York, New York, May 28, 29 and 30, 1957.

The program for the three day meeting will consist of papers and discussions dealing with the physiology and diseases of the thyroid gland.

THE NINTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE MEDICAL COLLEGE OF GEORGIA, AUGUSTA, GEORGIA, OCTOBER 21–25, 1957

The faculty will consist of twenty-two eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialities who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the Assembly.

For further information concerning the program and registration, write to *Dr. Robert B. Greenblatt, Department of Endocrinology, Medical College of Georgia, Augusta, Georgia.* Registration is limited to 100; tuition fee is $100.00. Rooms will be reserved for the students and faculty at the Bon Air Hotel. Residents and fellows will be admitted for $35.00.

NATIONAL INSTITUTES OF HEALTH

CIBA Pharmaceutical Products, Inc., Summit, New Jersey, has made available to the Endocrinology Study Section, National Institutes of Health (Bethesda 14, Maryland), its limited supply of *d,l*-aldosterone for biological and clinical investigation. This synthetic racemic material has one-half the biological activity of the natural *d*-aldosterone.

It is available to qualified investigators in the following forms:

d,l-aldosterone 21-monoacetate—purified sesame oil solution—200 micrograms/ml.
—purified sesame oil solution— 20 micrograms/ml.

d,l-aldosterone 21-monoacetate—95% ethanol solution—1 milligram/ml.

d,l-aldosterone (non-esterified)—95% ethanol solution—10 micrograms/ml.

The monoacetate will be distributed for biological work and the non-esterified form limited to use as a chromatographic standard. The alcohol solution of the monoacetate can be diluted for intravenous injections. Purified sesame oil can be obtained from CIBA for those who have occasion to dilute the oil solutions.

Requests should be made to Dr. R. T. Hill, Executive Secretary, Endocrinology Study Section, by letter briefly stating the exact amount wanted and the nature of the investigation.